

Cytotoxic and Allergenic Potential of Bioactive Proteins and Peptides

Rainer Hartmann^{1,*}, Jean-Michel Wal², Hervé Bernard² and Anne-Kathrin Pentzien¹

¹*Institute of Dairy Chemistry and Technology, Federal Dairy Research Centre, Location Kiel, P.O.Box 6069, D- 24121 Kiel, Germany and* ²*Laboratoire d'Immuno-Allergie Alimentaire, INRA-CEA de Saclay, F-91191 Gif sur Yvette, France*

Abstract: This review article deals with the assessment of cytotoxic and allergenic potential of bioactive proteins and peptides. It is evident that 'novel' foods or nutraceuticals containing bioactive proteins and peptides must fulfill their proposed "health claim". Furthermore, there is a need to assess their potential to exert adverse effects before they can be made widely available to consumers. A brief overview of compounds (i.e. proteins and peptides of animal and plant origin) and mechanisms involved in cytotoxic and allergenic (adverse) reactions is given along with some recent results obtained from ongoing studies.

There are numerous proteins and peptides of plant and animal origin that are known to exhibit cytotoxic effects. There is evidence that many cytotoxic compounds described in the literature exclusively affect malignant cells leading to the assumption that a cancer protective effect could exist for such bioactive proteins and peptides.

All the constituents that are responsible for the allergenicity of foods (as well as of pollens) are proteinaceous in nature. Some protein breakdown products, i.e. peptide fragments, may conserve part of the allergenicity of the native protein and thus can also be considered as allergens. The molecular basis of IgE recognition underlying cow's milk protein allergy is described. Some results from studies on volunteers fed caseinophosphopeptides or potentially hypotensive milk protein hydrolysates illustrate the major difference between allergenicity and immunogenicity. The data presented on the relationship between the structure of food proteins and peptides and their allergenicity shows the difficulty in trying to assess the "non-allergenicity" of products derived from an allergenic source, even if the process used involved extensive hydrolysis of the native protein(s). A 'weight of evidence approach' for assessing the potential allergenicity of a novel protein with no history of prior allergenicity is also presented with regard to the current EU Regulations.

Key Words: Cytotoxicity, allergenicity, antigenicity, bioactive peptides, proteins, apoptosis, sensitization, cancer, milk.

INTRODUCTION

In the search for bioactive peptides and proteins that are capable of producing health benefits when consumed as functional foods it is essential to verify the 'health claim' to be ascribed to the respective bioactive compound. This is a prerequisite for assigning a beneficial health effect to a product e.g. in advertising. Moreover, it is quite clear that any adverse effects such a compound might have must be investigated thoroughly and excluded prior to adding bioactive compounds to foodstuffs for general consumer availability. This means that a comprehensive safety assessment of the product should be performed to ensure that its consumption would not raise any risk to human health.

Adverse reactions to food are classically divided in toxic and non-toxic adverse reactions [1]. Toxic reactions including cytotoxicity occur in any individual of the general population provided that the dose of exposure is sufficient. In this case there is a clear relationship between the dose and the biological effect(s). The general concepts used in safety assessment (e.g. No-(Adverse)-Effect-Level and derived Acceptable Daily Intake) are then applicable. Non-toxic adverse reactions only occur in particular groups of the population or

even in predisposed individuals who have a particular genetic background and susceptibility to a certain food. This category comprises reactions that are mediated immunologically (e.g. allergy) and several other reactions depending on the biological mechanisms involved.

Adverse reactions that are not mediated immunologically also imply the so-called (food) intolerance phenomenon. The cause may be enzymatic (e.g. lactose-intolerance, which is due to lactase-deficiency occurring in large sections of the general population), pharmacologic (e.g. particular reactivity to bioactive substances which may be natural constituents of foods or to food additives) or most often unknown, combining several mechanisms.

This review gives an overview on the cytotoxic and allergenic potential of bioactive proteins and peptides of animal and plant origin that may be ingested e.g. as a functional food or in a pharmaceutical preparation.

CYTOTOXIC POTENTIAL OF BIOACTIVE PROTEINS AND PEPTIDES

There are numerous proteins and peptides of animal and plant origin that have been found to exhibit a bioactive potential beyond their mere nutritive value. These substances thus do not only supply the body with amino acids and C/N/O/H/S. In fact, they exhibit regulatory functions that very often lead to the formulation of health claims connected

*Address correspondence to this author at the Institute of Dairy Chemistry and Technology, Federal Dairy Research Centre, Location Kiel, P.O.Box 6069, D- 24121 Kiel, Germany; E-mail: rainer.hartmann@bfiel.de

with the consumption of such compounds or foods containing these proteins/peptides.

However, besides their beneficial potential they may also exert cytotoxic effects. Regardless of the relevance for the human system as a whole, knowledge of these cytotoxic effects are essential for a holistic evaluation of bioactive proteins and peptides contained within functional foods.

When evaluating cytotoxic proteins and peptides of plant and animal origin it is evident that some of these compounds seem to be most effective towards malignant cells. In fact, anticancer effects have been described and many plant extracts consisting of peptides/proteins are administered in adjuvant cancer therapy [2].

Principles of Cytotoxicity

As cytotoxic events can be exerted by numerous organic and inorganic agents affecting many different biochemical pathways, there are various cytochemical assay systems available to estimate the cytotoxic potential of a specific bioactive compound. Most of these assays also give an idea of the underlying mechanism or mode of action. There are several screening tests that rapidly measure unspecific cytotoxic effects (e.g. Neutral Red Assay, LDH Assay). Furthermore, there are assays that focus on specific biochemical markers or processes involved in programmed cell death (apoptosis) or DNA synthesis. In the search for beneficial or adverse effects on cell differentiation or intestinal epithelial integrity and functionality it is possible to quantify special enzymes, transepithelial electrical resistance (TEER) or paracellular permeability by investigating tight junctional routes of transport.

Although human studies and animal tests provide the most reliable data on toxicity including cytotoxicity, assays that measure cytotoxicity *in vitro* can give an idea of the (cyto)toxic potential of such compounds *in vivo* and may also help to understand the mechanism of action [3].

Assessment of Cytotoxicity

The term "cytotoxicity" implies all adverse effects a compound might have on the viability and reproducibility of cells, which means that a cytotoxic compound produces a toxic effect on cells. In this context, toxic effects can be described as either cytostatic or cell death related. This definition is rather general, and the effects mentioned can be very different depending on the cell type or population being observed and the principle of the assay applied i.e. its underlying biochemical principle.

When regarding cytotoxicity and its occurrence it is crucial to take into account the difference between the two major forms of cell death, apoptosis and necrosis.

Beginning with the initial observations of changes in nuclear morphology that distinguish apoptosis from necrotic cell death, the field of cell death research has rapidly expanded, generating a wealth of knowledge about the genetic and biochemical pathways that control this process (reviewed in [4]). Several assays for detection of apoptosis have also been developed during this past decade, some of which are applicable to cytochemical analysis [5].

Table 1 gives a brief summary of different methods which are frequently applied to determine cytotoxicity.

Assessment of Non-Specific Cytotoxic Effects

The LDH leakage assay is based on the measurement of LDH activity in the extracellular medium. The loss of intracellular LDH and its release into the surrounding culture medium is an indicator of irreversible cell death due to cell membrane leakage [6, 7]. The LDH assay is fast, reliable and simple to carry out. LDH activity is detected by the amount of NADH that is produced when lactate is converted to pyruvate by LDH, as NADH can easily be quantified by its absorbance at 340 nm.

The neutral red (NR) assay is also applied to measure cellular death as a response to a cytotoxic agent. NR is a weak cationic dye that diffuses readily across plasma and organelle membranes, accumulating in the lysosomes. Most protocols applied are based on the NR uptake assay first described by Borenfreund and Puerner [29]. Loss of membrane integrity induced by test agents results in decreased retention of NR (quantification of NR at 540 nm). Damaged or dead cells thus appear decolorized in comparison with healthy control cells. Similarly, lower absorbance after NR extraction is an indication of reduced cellular viability [30].

Cytotoxicity assays are very often based on tetrazolium salts that are converted to the corresponding formazan dye by cellular reductases of living cells [31]. The prototype is MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), a water-soluble salt that is cleaved by mitochondrial succinate reductase (NAD(P)H dependent) to a water insoluble formazan that accumulates in living cells. Prior to photometric detection the dye must be solubilized. This inconvenience led to the development of tetrazolium salts whose corresponding formazan dyes are soluble (XTT, WST-1) and non-cytotoxic (WST-1) [12].

Other assays measure markers of cellular vitality such as acid phosphatase [32] and alkaline phosphatase activity [33, 34] or use different stains for colorimetric cell quantitation (Janus green, crystal violet, Coomassie blue, methylene blue, naphthol blue-black, naphthol yellow S, May-Grünwald-Giemsa, sulforhodamine B) [35].

In principle, all viability assays based on dye formation or uptake assume a linear relationship between number of living cells and the amount of dye measured.

In addition, staining of dead cells can also be performed using different dyes. The Trypan Blue (TB) exclusion assay is based on the fact that TB only penetrates dead cells exhibiting membrane lesions. The colored dead cells can easily be observed by light microscopy. Quantification of cytotoxic effects is easily performed using fluorescent DNA staining agents like propidium iodide, ethidium bromide, and 7- Δ AD. Fluorescence is detected either by fluorescence microscopy or cytometry [36, 37, 38].

Assessment of Programmed Cell Death (Apoptosis)

Apoptosis, or programmed cell death, is a highly regulated process responsible for orderly cell removal, and thus acts in concert with cell division to control cell numbers over time [39]. Apoptosis is an essential mechanism to eliminate

Table 1. Examples of Cytochemical Assays to Determine and Characterize the Occurrence of Cytotoxic Effects *In Vitro*

Cellular Event Measured	Assay Name	Biochemical Basis	Method of Measurement or Detection	Ref.
Necrosis/ membrane lesions	LDH Assay	Conversion of lactate to pyruvate in presence of LDH released through membrane lesions	Concomitantly, NAD is reduced to NADH, which results in a change in absorbance at 340 nm	[6,7]
	Neutral Red Assay	Neutral red can only be retained by viable cells	Measurement of NR absorbance at 540 nm	[8,9]
	DNA staining of dead cells	Fluorescent/non-fluorescent dyes penetrate membranes of dead cells and bind to DNA	Trypan blue (light microscopy); propidium iodide, ethidium bromide, 7-AAD etc. (fluorescence)	[10]
Cell viability	MTT, XTT, WST-1	Cleavage of various tetrazolium salts by reductases of living cells	Reductases cleave tetrazolium to formazan, increased absorbance at 578/490/540 nm (MTT/XTT/WST-1)	[11,12]
Apoptosis	ISEL, TUNEL	Free DNA strand ends occur during apoptosis	Labeling 3'-OH ends of single stranded DNA	[13]
	Cell Death Detection ELISA	Apoptotic DNA cleaved into mono- and oligonucleosomes by endonucleases	Mono-/oligonucleosomes detected in sandwich ELISA: anti-histone-biotin + Anti-DNA-HRP conjugates, ABTS	[14]
	Annexin V	Externalisation of phosphatidylserine (PS) to the outer membrane leaflet	PS is detected by annexin V probe, counterstaining of necrotic cells by trypan blue or propidium iodide	[15]
	Caspase Assay	Activation of caspases following death signals	Absorbance/fluorescence of dye released by active caspases	[16,17]
	Apoptotic DNA Ladder Assay	DNA is cleaved by endonucleases to multiples of 180 bp subunit	Cell lysis, DNA extraction, resolving DNA on an agarose-gel by electrophoresis, apoptosis is indicated by a "ladder"	[18]
Cell proliferation (cell number, DNA synthesis)	Microscopy/cell counting	Proliferation results in augmentation of cell number in the sample	Increase in cell number is observed by light microscope or cell counting in counter or flow cytometer	[19]
	Protein Assay Lowry/Bradford	Proliferation results in augmentation of protein content in the sample	Cu^{2+} is reduced to Cu^+ by primary amines and gives a blue dye	[20]
	BrdU assay	Incorporation of BrdU instead of thymidine into newly synthesized cellular DNA	Detection of incorporates BrdU by antibody versus BrdU in an ELISA or FACS protocol	[21]
	^3H -assay	Incorporation of ^3H -thymidine instead of 'cold' thymidine into newly synthesized DNA	Measurement of ^3H -activity in the sample using liquid scintillation counting	[22]
	MTT, XTT, WST-1	Increased cell number means more metabolically active reductases in the sample	Reductases cleave tetrazolium to formazan, increased absorbance at 578/490/540 nm (MTT/XTT/WST-1)	[23]
Functional integrity of cell membrane or cell monolayer	Alkaline phosphatase assay	Brush border ALP expressed in differentiated Caco-2 cells cleaves phosphoester bonds	Measurement of alkaline phosphatase activity using Fluorophos (quantitation of the product Fluoroyellow)	[24,25]
	TEER TJ function	Tight junctions formed during differentiation of intestinal epithelial cells (e.g. Caco-2)	Resistance is measured by applying a low current using two electrodes and an ohm-meter	[26,10]
	DPP IV assay	DPP IV from brush border cleaves X-pro-, X-Ala-, X-Lys-oligopeptides	Ala-Pro-p-nitroanilide is cleaved to p-nitroanilin and Ala-Pro; p-nitroanilin is detected by colorimetry	[27]
	Tight Junction Permeability Assay	Different compounds are transported through tight junctions of differentiated Caco-2 cells	Measurement of e.g. ^{14}C - or ^3H -mannitol (182 Da) or FITC-Dextran (3 kDa) transported through monolayer	[28]

unwanted cells during the development and homeostasis of multicellular organisms [40, 41].

Necrosis, by the other hand, is a passive, pathological and accidental process that is not very well regulated. Morphological criteria distinguish necrotic from apoptotic cells that show chromatin condensation at the nuclear periphery, chromatinolysis, cellular shrinkage, disassembly of nuclear scaffold proteins, and formation of apoptotic bodies [42].

Two main pathways can lead to apoptosis: the intrinsic (or stress) and the extrinsic (or death receptor) pathways. The extrinsic pathway involves activation of a plasma membrane receptor of the death receptor superfamily (Fas/CD95) and an adaptor protein (e.g. FADD), leading to the receptor-proximal recruitment of a caspase activation complex. The intrinsic pathway critically relies on mitochondrial outer membrane permeabilization (MOMP), which causes bio-

energetic failure and directly releases soluble proteins contained in the mitochondrial intermembrane space. These molecules include cytochrome c, apoptosis induction factor (AIF), endonuclease G (endo G), Omi/HtrA2 and Smac/DIABLO [43]. Cytochrome c, once in the cytosol can interact with other proteins in a sequence of events that culminate in biochemical changes such as significant caspase(s) activation, cell volume loss, exposure of phosphatidylserine and nucleosomal DNA fragmentation [44]

Assessment of Cell Proliferation

Cell proliferation results in an augmentation of cell number in a sample. This can be determined e.g. by light microscopy or in cell suspensions by conventional cell counters or flow cytometers [19].

Moreover, cell proliferation can be determined indirectly using assays that measure viable cells in the treated sample. An increased cell number leads e.g. to higher amounts of dyes produced by living cells (e.g. formazan) or of dyes only retained by living cells (e.g. NR), resulting in an increased color intensity.

Cell proliferation is always accompanied by DNA replication/synthesis. The conventional tritiated thymidine (^3H -TdR) incorporation assay was considered as the 'gold standard' for the assessment of cell DNA synthesis for many years. However, the ^3H -TdR incorporation assay has several disadvantages which have prompted the development of non-radiolabeling proliferation assays such as 5-bromo-2-deoxyuridine (BrdU) ELISA [22] or detection of BrdU-incorporation by flow cytometry [45]. BrdU is a thymidin analogue and is incorporated instead of thymidine into newly synthesized DNA [46]

Another possibility to monitor cell proliferation is to determine cell cycle associated enzymes and proteins, e.g. cyclins and cyclin-dependent kinases (cdk) or elements of the signal transduction cascade related to mitogen-activated kinases (MAPK) [47].

Other assays used to quantify cell proliferation are based on direct dye reactions with cellular protein, assuming a linear relationship between cell number and dye signal. Most of these methods go back to the method described by Lowry in 1951 [20] and Bradford in 1976 [48].

Assessment of Effects on Epithelial Functions of Cultured Intestinal Cell Monolayers

Morphological and functional integrity of the human enterocytes is crucial for all processes involving the digestion and absorption of ingested nutrients. Besides, epithelial cell monolayers represent a barrier for toxins and antinutrients. Cytotoxic food proteins and peptides can affect this gate-keeping function of enterocytes.

An accepted model system for the human enterocyte is the Caco-2 and HT29 model, as - with ongoing differentiation - these colon carcinoma cells exhibit morphological and functional similarities to non-malignant human enterocytes (cell polarization, expression of brush border enzymes, formation of tight junctions) [49].

Tight junctions(TJ) between intestinal epithelial cells play an important role in the regulation of transport of organic and inorganic compounds from the gut lumen towards blood circulation [50]. To date, several integral membrane proteins, including occludin, claudin(s) and the junctional adhesion molecule-A (JAM-A), have been localized to functional TJ structures [51]. TJ membrane proteins interact with scaffold proteins (e.g. ZO-1) to connect them with various signal transduction and transcriptional pathways involved in the regulation of TJ function [52].

TJ function can be determined by the measurement of transepithelial electrical resistance (TEER) using a two-compartment cell culture system, separating the upper (apical) part of the epithelium from a basolateral compartment mimicking the cellular sites facing blood circulation. One main advantage of the TEER assay is that it is non-destructive and changes in TEER i.e. tight junction permeability can be monitored over a long period of time [26]. Fig. (1) gives an idea of the measurement principle and some results from Caco-2 cell TEER measurement after 4 h exposure to a commercially available lactalbumin preparation (Lactalbumin 70; MILEI GmbH, Stuttgart/D)¹.

Integrity of cultured monolayers is also detectable by carrying out transport studies using water-soluble reference compounds that can pass through the tight junction channels between the cells (e.g. radio-labeled mannitol, phenol red, Lucifer Yellow, or fluorescein; M_r : 182, 354, 57, 332 Da, respectively). Quantification is then made by detecting radioactivity, absorbance, fluorescence or luminescence of the solution taken from the compartment opposite to the side where test compounds were applied [53].

Another approach to detect cytotoxic effects on epithelial structures is to determine changes in activity of brush border associated enzymes of intestinal epithelial cells. Caco-2 and HT29 cells have also shown to represent a suitable model for such studies. Table 2 gives an overview on the diversity of brush border associated hydrolases of human enterocytes. Frequently used assay systems determine activity of lactase, sucrase-isomaltase, dipeptidyl-aminopeptidase IV and alkaline phosphatase in cultured epithelial cells (e.g. Caco-2) or extracts thereof [27, 54, 55].

Dipeptidyl-aminopeptidase IV (DPP IV, EC 3.4.14.5; CD 26) is a specific marker of mature enterocytes and differentiated Caco-2 cells. DPP IV is synthesized within the cell as a dimeric glycoprotein and is subsequently integrated in the apical cell membrane (brush border), with its active site being situated in the gut lumen [56]. Thus DPP IV activity can be measured without prior cell lysis, simply by adding a specific substrate, e.g. Ala-Pro-p-nitroanilide. DPP IV catalyzes cleavage of the substrate into the Ala-Pro dipeptide and the yellow-colored p-nitroaniline. The color intensity can be measured and directly correlates with cell number. Fig. (2) presents some results obtained with Caco-2 cells after 4 h exposure to a commercially available Lactalbumin (LA 70) preparation².

¹ Pentzien AK: unpublished results

² Pentzien AK: unpublished results

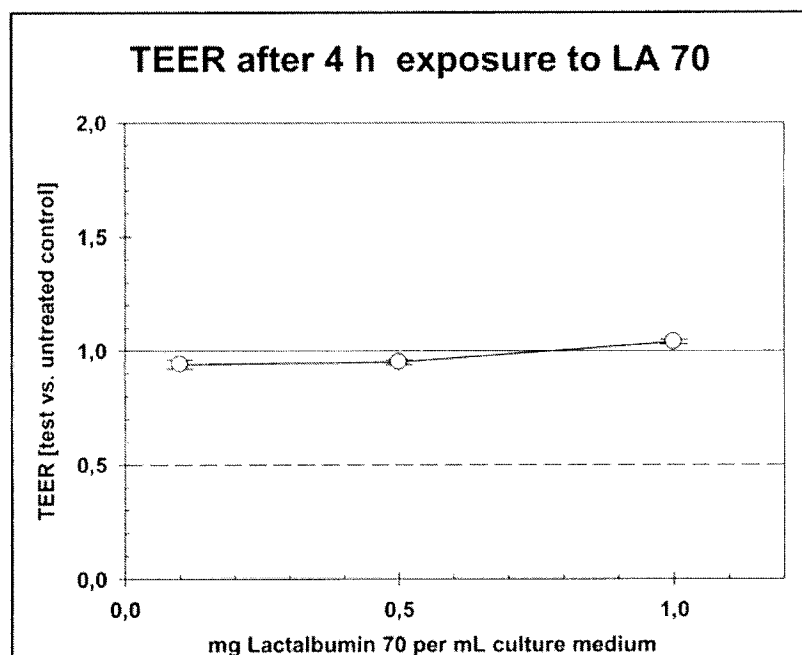
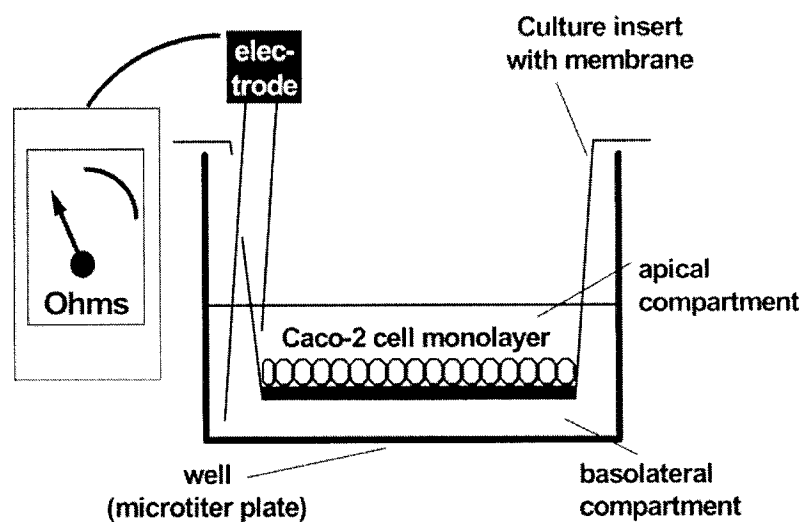


Fig. (1). Transepithelial electrical resistance: principle of measurement (above); example of TEER assay (down): TEER of Caco-2 cells exposed to Lactalbumin 70 (LA 70) for 4 h is comparable to TEER of untreated control cells.

Bioactive Proteins and Peptides Possessing Cytotoxic Potential

Studies have been undertaken to explore the utility of cell culture techniques in testing plant and animal products for toxicity.

Tables 3 and 4 give an overview on bioactive proteins and peptides of plant and animal origin, respectively, that exhibit a specific cytotoxic potential *in vitro*.

Proteins and Peptides of Animal Origin

Literature dealing with cytotoxic effects exerted by proteins and peptides of meat, egg and fish is very scarce. Only carnosine and anserine contained in meat seem to exhibit

significant cytotoxic effects towards human cell systems, but the relevance of these results for humans is not yet clear.

Among the bioactive peptides of animal origin the peptides derived from milk have been subject of extensive studies internationally. Consequently, most data on the cytotoxic potential of proteins and peptides of animal origin have been collected for various intact milk proteins and associated peptides. As for the beneficial properties of these compounds, enzymatic hydrolysis is a prerequisite for releasing the active cytotoxic regions encrypted in the intact proteins [57].

The numerous bioactive effects that have been assigned to milk protein-derived peptides have been reviewed elsewhere [58].

Table 2. Intestinal Brush Border Associated Hydrolases

Enzyme	Substrates and Properties	Products
Endopeptidases:	Hydrolysis of internal peptide bonds:	
enterokinase	of trypsinogen (initiation of luminal digestion)	trypsin
neutral endopeptidase (EC 3.4.24.11)	at hydrophobic amino acids of α -casein, insulin etc.	peptides
Oligopeptidases:	Hydrolysis of N-terminal peptide bonds:	
aminopeptidases (AP) AP N (EC 3.4.11.2) AP A (EC 3.4.11.7) AP P (EC 3.4.11.9) AP W (EC 3.4.11.16)	AP are metalloenzymes (Zn, Ca, Co). AA-oligopeptide of neutral amino acids AA-oligopeptide of acidic amino acids Pro-oligopeptide X-Trp, X-Trp-oligopeptide	amino acids di-, tripeptides
dipeptidyl-AP IV (DPP IV; EC 3.4.14.5)	X-Pro-oligopeptide X-Ala-oligopeptide X-Lys-oligopeptide DPP IV is a serine protease.	X-Pro, X-Ala, X-Lys, oligo-, dipeptides
Carboxypeptidases (CP): dipeptidyl-CP I; EC 3.4.15.1	Hydrolysis of C-terminal peptide bonds: angiotensine, oligopeptide-Pro synonyme: angiotensin-converting enzyme, peptidase P	dipeptides
γ -glutamyl-transpeptidase EC 2.3.2.2	peptides with bound γ -glutamyl, e.g. glutathion	peptides, γ -glutamyl-AA
folate conjugase; EC 3.4.19.9	polyglutamyl folate	folic acid
CP M; EC 3.4.17.12	peptide-Lys, peptide-Arg	basic amino acids
Dipeptidases:	Hydrolysis of dipeptides into AA	
Zn-stable Asp-Leu peptidase	dipeptides, esp. Asp-Leu	amino acids
Gly-Leu peptidase	dipeptides, esp. Gly-Leu metalloenzyme (Zn)	amino acids
membrane dipeptidase	dipeptides (glutathione conjugate)	amino acids
Other Hydrolases:		
alkaline phosphatase EC 3.1.3.1	phosphate monoester	phosphate + alcohol
sucrase – isomaltase EC 3.2.1.48	hydrolysis of 1,6- α -D-glucosidic linkages in some oligosaccharides produced from starch and glycogen by EC 3.2.1.1 (α -amylase), and in isomaltose.	glucose
lactase EC 3.2.1.23	lactose	glucose + galactose

Proteins and Peptides of Plant Origin

Antinutrients commonly found in plant foods have both beneficial and adverse health effects. For example, phytic acid, lectins, phenolic compounds (tannins), saponins and enzyme (amylase and protease) inhibitors have been shown to reduce the availability of nutrients and cause growth inhibition, while phytoestrogens and lignans have been linked with infertility problems. However, phytic acid, lectins, phenolic compounds, amylase inhibitors and saponins have also been shown to reduce blood glucose and insulin responses to starchy foods and/or the plasma cholesterol and triglycerides. In addition, phytic acid, phenolics, saponins, protease inhibitors, phytoestrogens and lignans have been related to reduced cancer risks which implies a certain cytotoxic potential to at least malignant cells.

Cereals, legumes, and tubers consumed by humans may thus contain significant amounts of antinutritional or cytotoxic components such as phytates, tannins, cyanogenic glycosides, oxalates, saponins, lectins, and inhibitors of enzymes such as α -amylase, trypsin, and chymotrypsin (reviewed in [81]).

This section of the review focuses on proteins and peptides of plant origin that have turned out to exert cytotoxic effects *in vitro* and also to some extent *in vivo*.

A variety of proteins and peptides comprising lectins [82, 83], ribosome-inactivating proteins and peptides, α -amylase inhibitors [84], arcelins [85], antifungal proteins and peptides, protease inhibitors [86], and napins [87] are produced by seeds. It has been reported that some of these proteins exhibit exploitable activities such as immunomodulatory, anti-

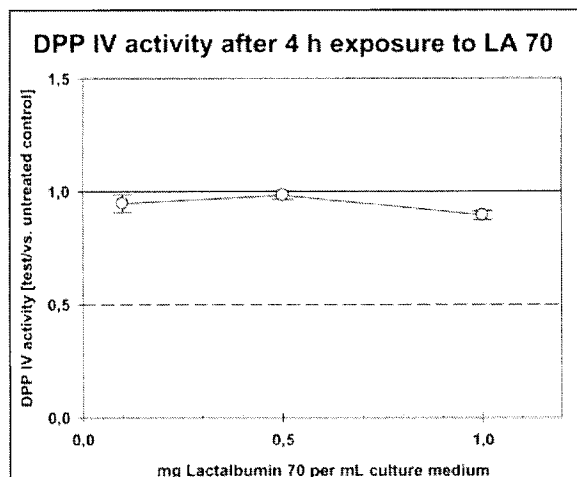


Fig. (2). No relevant changes in DPP IV activity of differentiated Caco-2 cells were determined after 4 h exposure to Lactalbumin 70 (LA 70).

fungal, antiproliferative, anti-insect and HIV-1 enzyme-inhibiting activities [88]. The following three sections deal with proteins/peptides which have been intensively studied that play a specific role for food ingested by humans and, comprising plant defensins, lectins and protease inhibitors.

A prototype of a plant – although not for food usage – exhibiting cytotoxic compounds that are ascribed a certain beneficial effect is the mistletoe (*Viscum album* L.). Two of its constituents that are highly effective are viscotoxins and mistletoe lectins. In fact, already in 1920, mistletoe extracts were proposed for adjuvant cancer treatment by Rudolf Steiner and Ita Wegmann, founders of anthroposophic medicine [89]. However, published results of cancer treatment studies are contradictory [90, 83].

Plant Defensins, Gamma-Thionins

All living organisms, ranging from microorganisms to plants and mammals, have evolved mechanisms to actively defend themselves against pathogen attack. Besides complex strategies for adaptive immune responses, principles of innate immunity exist, involving, among others, the production of defense peptides, the so-called defensins. The structural properties of defensins seem to be conserved between plants,

Table 3. Examples of Proteins and Peptides of Animal Origin that have Shown to Exhibit a Certain Cytotoxic Potential *In Vitro*

Origin	Name of Active Compound	Chemical Characteristics	Cytotoxic Effect <i>In Vitro</i>	Ref.
Milk	Lactoferrin	iron-binding glycoprotein, polypeptide chain of about 690 amino acid residues	reduces lung colonization (experimental metastasis) by B16-F10 melanoma cells, inhibits GLP-2 mediated epithelial proliferation, inhibits lectin-mediated proliferation of lymphocytes	[59,60,61]
	Lactoferricin	25-residue-fragment of lactoferrin, released by pepsin-proteolysis	antitumour action e.g. versus fibrosarcoma, B16F10 melanoma and C26 colon carcinoma cells, hemorrhagic necrosis in tumor sections	[62,63]
	Lactoferricin-derived peptides	11-residue linear peptide of lactoferricin B	peptides showed cytotoxic action in an anti-cancer assay against neuroblastoma cell lines.	[64]
		f(17-38) of bovine lactoferrin	inhibits cell proliferation through apoptosis	[65]
	Caseinophosphopeptides	f(59-79) of α_1 -casein, 5 SerP f(1-25) of β -casein, 4 SerP [66]	significantly inhibited the concanavalin A-induced proliferation of mouse spleen cells and rabbit Peyer's patch cells	[67]
	β -Caseinophosphopeptide	f(1-25) of β -casein, 4 SerP	inducing apoptosis in some malignant cells	[68]
	α -Casecidins	peptides from trypsin-digest of bovine α_1 -casein, fragments 1-3 (Arg-Pro-Lys), 101-103 (Leu-Lys-Lys) and 104-105 (Tyr-Lys)	cytotoxicity (necrosis) towards healthy mouse T and B cells and human leukemic T and B cell lines	[69]
	κ -Casecidins	f(17-21) from bovine κ -casein, cytotoxic motif Phe-Phe-Ser-Asp-Lys	cytotoxic towards mouse spleen cells, bovine milk cells and human cell lines, esp. T cells	[70,71]
	κ -Caseinoglycopeptides	pepsin or rennin digest	induction of apoptosis in lymphocytes, Jurkat cells, suppression of lymphocyte proliferation	[72]
Meat	α -Lactalbumin	component of lactose synthase, esp. higher M_r (20/30 kDa) forms of the α -lactalbumin are cytotoxic	inhibits proliferation of intestinal cell line IEC-9, Caco-2 and HT29, induces apoptosis in lymphoid cells	[73-77]
	Carnosine	β -alanyl-L-histidine	slight DNA damage in human lymphocytes, killing transformed cells	[78,79]
	Anserine	3-methyl-carnosine	slight DNA damage in human lymphocytes	[80]

Table 4. Examples of Proteins and Peptides of Plant (Food) Origin that have Shown to Exhibit a Certain Cytotoxic Potential *In Vitro*

Origin/Source	Name	Chemical Characteristics	Cytotoxic Effect <i>In Vitro</i>	Ref.
Mistle toe (<i>viscum album</i>)	viscotoxins	cysteine rich, 5 kDa, defensin-type	induction of necrosis in many cell types	[103,104]
	mistle lectins	consisting of A and B chain	induction of apoptosis, inhibition of DNA synthesis	[105]
Castor oil plant (<i>ricinus communis</i>)	ricin	ribosome-inactivating A chain and galactose-binding B chain (lectin)	inhibition of cell proliferation, e.g. in dendritic cells and HeLa cells	[106,107]
Cherimoya-Fruit (<i>annona cherimola</i> , <i>annona glauca</i>)	cherimolacyclopeptides, glaucacyclopeptide	small, cyclic peptides	cytotoxic activity against the KB (human nasopharyngeal carcinoma) cell culture system	[108,]
Rapeseed (<i>brassica napus</i>)	napin	4.5 kDa subunit and 10 kDa subunit, disulfide-linked [109,110]	RIP, inducing apoptosis	[87,111]
Cabbage (brassica alboglabra, b. parachinensis)	napin-like peptides	~14.5 kDa	antiproliferative activity against leukemia L1210 cells, RIP	[88,112]
Runner Beans (<i>phaseolus coccineus</i>)	phaseococcin coccinin	N-terminal sequence homologous to those of defensins	inhibition of proliferation in leukemia cell lines HL60 and L1210	[113,114]
Jack Beans (<i>canavalia ensiformis</i>)	concanavalin-A	lectin, binds to mannose/ glucose residues	inhibition of HT29 cells (in higher conc.)	[115,116]
	canatoxin	isoform of Jack Bean urease	bioactivity independent of ureolytic activity	[117]
Common Beans (<i>phaseolus vulgaris</i>)	vulgarinin	~7 kDa peptide, defensin-type	inhibited proliferation in leukemia cell lines L1210 and M1 and breast cancer cell line MCF-7.	[118,119]
Tepary Beans (<i>ph. acutifolius</i>)	protease inhibitor	Ser proteinase inhibitor, 7.1 kDa, BB class	<i>in vitro</i> inhibition of HeLa cell growth	[120,121]
Pea (<i>pisum sativum</i>), Lentil (<i>lens culinaris</i>)	protease inhibitor	8 – 12.8 kDa, BB class	inhibition of HT29 cell growth	[122]
Pea (<i>pisum sativum</i>)	<i>pisum sativum</i> defensin I	46 amino acid residue plant defensin	inhibits proliferation, RIP	[119,123]
Soybean (<i>glycine max</i>)	Bowman-Birk Inhibitor	serine proteinase inhibitor, 8 kDa	cytotoxic activity on prostate cancer cells and HT29 cells; <i>in vitro</i> inhibition of breast cancer cells	[124-126]
	lunasin	43-AA peptide, cell adhesion motif – RGD, followed by 8 Asp	stops proliferation; induces apoptosis in malignant cells, inhibits acetylation of core histones.	[127,128]
Buckwheat (<i>fagopyrum esculentum</i>)	buckwheat PI	5 to 7 kDa, BB class	inhibition of leukemia cells	[129]
Wheat and other <i>graminae</i>	rice bran agglutinin	36 kDa, 2 identical 18 kDa-subunits, each with of 2 chains (10/8 kDa)	growth arrest of human monoblastic leukemia U937 cells by G2/M phase cell cycle arrest	[130]
	wheat germ agglutinin	26 kDa lectin	apoptosis/necrosis in human leukaem. cells+PBMC	[131,132]
	gliadine-derived peptides	various peptides in peptic-tryptic digest	inhibition of Caco-2 cell DNA/RNA synthesis	[133,134]
	gamma-thionins, defensins	defensins from wheat, barley, ~5 kDa, homologous to viscotoxins	inhibition of growth of different mammalian cells	[103,135]

Abbreviations: (AA) amino acid(s); (BB) Bowman-Birk; (PI) protease inhibitor; (RIP) ribosome inactivating protein.

invertebrates and vertebrates, including the highly basic character and disulfide bridges. The size and spatial orientation of the triple-stranded β -sheet in mammalian defensins is comparable to that in plant defensins [91, 92].

Gamma-thionins are small cysteine-containing, amphipathic plant proteins found in seeds and vegetative tissues of a number of plant genera. Many of these have been shown to

be toxic to microorganisms such as fungi, yeast, and bacteria and also to mammalian cells. It has been suggested that thionins are present in seeds to protect them, and the germinating seedling, from attack by phytopathogenic microorganisms. There is evidence that the generalized toxicity of gamma-thionins is due to their ability to generate ion channels in cell membranes, resulting in the dissipation of ion

concentration gradients essential for the maintenance of cellular homeostasis [93].

Protease Inhibitors

Protease inhibitors (PI) probably were the first compounds from dietary origin to be tested for cytotoxic effects towards cancer cells [94], despite of their antinutritional role with regard to the digestive tract [81]. PIs are widely distributed in living organisms; in particular, plant seeds are known to be rich in these compounds. Different types of PIs have been found and inhibitors of the *Kunitz*- and the *Bowman-Birk*-Type have been studied intensively. Bowman-Birk-Inhibitor (BBI) was initially isolated from soybean and was later found in other plants such as rice, potatoes and beans. The Bowman-Birk Inhibitor (BBI) belongs to the serine-protease inhibitor family, harboring a unique function of independently inhibiting trypsin and chymotrypsin activity [95].

Soybean Bowman-Birk inhibitor (SBBI) has been reported to act as an anticancer agent and has therefore led to a growing interest in BBI and BBI-like molecules among the medical community [96].

Lectins

In 1888, Peter Hermann Stillmark (1860-1923) completed his doctoral thesis at the Dorpat University in Tartu/Estonia "Ueber Ricin, ein giftiges Ferment aus den Samen von *Ricinus comm. L.* und einigen anderen Euphorbiaceen" [97]. He succeeded in isolating a poisonous protein component from castor beans that he named "Ricin". That event is internationally recognized as the birthday of a new branch of science called lectinology.

Ricin again became very famous in the autumn of 1978, when a Russian civil service agent murdered the Bulgarian author Georgi Markov in his exile in London, injecting ricin into his leg by means of a specially prepared umbrella tip.

Plant lectins, a unique group of proteins and glycoproteins with potent biological activity, occur in foods like wheat, corn, tomato, peanut, kidney bean, banana, pea, lentil, soybean, mushroom, rice, and potato. Thus, dietary intakes by humans can be significant. Many lectins resist digestion, survive gut passage, and bind to gastrointestinal cells and/or enter the circulation intact, maintaining full biological activity. Several lectins have been found to possess anticancer properties *in vitro*, *in vivo*, and in human case studies; they are used as therapeutic agents, preferentially binding to cancer cell membranes or their receptors, causing cytotoxicity, apoptosis, and inhibition of tumor growth. These compounds can become internalized into cells, causing cancer cell agglutination and/or aggregation [83].

In general, lectins are carbohydrate-binding proteins that are widely distributed among different living organisms. They are defined today as 'carbohydrate-specific binding proteins of non-immune origin that agglutinate cells and/or precipitate glycoconjugates'. These compounds can be extremely toxic, and they are denatured by heating [98]. The cell agglutinating properties come from the fact that lectins have two or more binding sites for a carbohydrate moiety leading to binding to at least two adjacent cells [99].

Lectins can also exhibit ribosome-inactivating activities and thus inhibit protein synthesis. Ricin, for example is a dimeric protein consisting of two chains (A/B), connected by one disulfide bond. The A chain (ricin toxin A, RTA) represents a rRNA N-glycosylase (EC 3.2.2.22), hydrolyzing the N-glycosidic bond at one specific adenosine on the 28S rRNA (a4324 in mammals). The B chain (RTB) is the lectin component, a two-domain protein, able to bind two galactose residues (Fig. (3)) [100, 101]. Recent studies suggest that ribosome-inactivating proteins are also capable of directly inducing cell death by apoptosis [102].

ALLERGENIC POTENTIAL OF BIOACTIVE PROTEINS AND PEPTIDES

Some of the proteins previously mentioned as exerting a cytotoxic effect may also act as allergens by a completely different mechanism.

Principles of Food Allergy

Immune-mediated adverse reactions include non-IgE-mediated (e.g. inflammatory diseases that can be consecutive of delayed type hypersensitivity) and IgE-mediated reactions (immediate type hypersensitivity). Although the prevalence of non-IgE-mediated adverse reactions seems to be increasing, particularly in children, the denomination (food) allergy generally refers to IgE mediated, immediate type hypersensitivity. Depending on the severity of the symptoms, the word anaphylaxis can also be used.

Allergy is a pathological deviation of the immune response to a particular substance, which affects only a few individuals who have a predisposed, "atopic", genetic background.

Food/drug allergy consists of two separate phases: first *sensitization* where no symptoms occur, and later *elicitation* with clinical manifestations. When ingested, the allergen i.e. the sensitizing food or a particular food constituent is to some extent destructured then degraded by digestive enzymes, absorbed by the gut mucosa, processed and presented to the immune cells. In allergic patients, this results in a deregulated synthesis of so-called reagenic antibodies of the immunoglobulin E (IgE) class that are produced in excess by lymphocytes. The specific IgE circulate in the organism and bind to the membrane of blood basophils and tissue mast cells. These cells produce and may store allergy mediator substances such as histamine, prostaglandins, leucotrienes, and others. Subsequent contact of the immune system with the same allergen, or with another related molecule sharing some common immunoreactive structures (termed epitopes) with the parent sensitizing allergen, cause more specific IgE to be produced and to bind to basophils and mast cells. Finally, the allergen (as the intact molecule or fragments comprising at least 2 IgE binding sites) bind and cross link specific IgE present on the membrane of basophils and mast cells which triggers cell degranulation and release of pharmacologically active mediators, causing the onset of the various symptoms of the allergic reaction. The clinical manifestations are not limited to the gastrointestinal tract but may also be cutaneous and respiratory. They may be generalized resulting in an anaphylactic shock which may be fatal. In general the severity of the allergic reactions cannot be fully

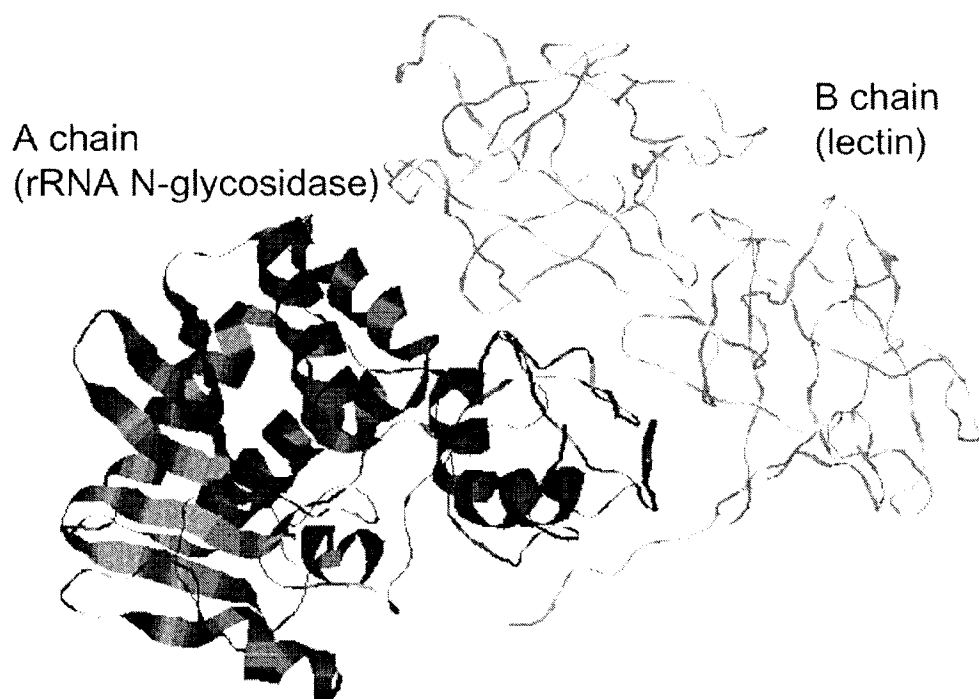


Fig. (3). Structure of Ricin; A chain (left), B chain (right); image obtained with program RasMol v. 2.6, starting from PDB-ID 2AA1.

predicted and depends on several factors including the genetic susceptibility of the individual, the physiological state, the nature, dose and presentation of the allergen. Although a certain relationship exists between the level of exposure to an allergen and its potential to induce a sensitization or to elicit a reaction, there is no clear and definite relationship to predict the likelihood and severity of an allergic reaction. For a given allergic individual, a threshold dose exists below which an allergic reaction is unlikely to be elicited. However this threshold is highly variable for each individual, depending on his/her genetic susceptibility and environmental factors and depending on the nature and presentation of the allergen. The inter individual variability is also very high which so far has precluded to consider threshold doses a reliable tool for management of the allergy risk. Avoidance of the offending foods is the only practical means available to consumers who have allergies or intolerances.

Allergenicity may designate both the sensitizing potential which is the capacity of a compound to induce the synthesis of specific IgE by a genetically predisposed (i.e. atopic) individual and/or the eliciting potential which is the capacity to trigger the release of pharmacologically active mediators then provoke an allergic reaction in already sensitized consumers. Both risks should be evaluated when assessing the human safety of a (novel) functional food or novel food ingredient with enhanced nutritional value and/or health claim. However, so far the risk assessment process has essentially focused on the second aspect, i.e. the risk for allergic consumers to elicit severe reactions by eating a food containing a constituent to which they are sensitized, because they are not aware that this constituent may be present.

In terms of frequency, food intolerances represent the vast majority of adverse food reactions. However food al-

lergy has become a major health concern in developed countries because the prevalence appears to have much increased during the past 10 years. The severity of the clinical manifestations of food allergy, including fatal anaphylactic shocks, apparently also has increased as well as the number of incriminated foods. In recent surveys, up to 25% of adults believed that they had a food allergy. This perception gives an over estimation of the prevalence of food allergy which cannot be accurately estimated because of the lack of appropriate epidemiological studies performed in different countries. However based on clinical studies around 6-8% of children are considered suffering of food allergic disorders during the first 3 years of life, in most cases cow's milk and hen's egg are the responsible foods. Most of those children outgrow their allergy. Food allergies are observed more often in children than in adults. It is estimated that 2-3% of the adult population may suffer of food allergy in the USA and European Union [136-139].

Allergenic Constituents of Foods and Allergenic Structures (Epitopes)

The term "allergenic" food refers both to the complex whole food and to the chemically defined compounds that are responsible for allergenicity, i.e. the proteins. Some protein breakdown products, i.e. peptide fragments, may retain part of the allergenicity of the native protein and thus can also be considered as allergens. Food allergens are generally glycoproteins of molecular weight ranging from ca. 12 to 30-40 kDa as well as polymers formed by those proteins, but each protein constituent of a food may potentially be allergenic. Food allergens involve common proteins with highly conserved structures, which generally correspond to important biological activities and functions that are vital for the plant (metabolic, protective (defense) or storage function).

They also generally are proteins with a globular compact structure stabilized by hydrogen and disulphide bonds as shown on 2-D and 3-D structure of α -lactalbumin and β -lactoglobulin, major allergens of the whey fraction of cow's milk (Fig. (4) and (5)). They are often glycosylated, stable to processing and resistant to proteolysis by digestive enzymes. Allergenicity of a given complex food is very rarely due to a single protein component but rather to numerous different proteins, which constitute the "allergen repertoire" of the food. Due to the diversity and variability of the human IgE response, patients allergic to this food do not always recognize all of the allergenic proteins. Those allergens that are recognized by more than 50% of a population of patients allergic to the food are called major allergens. This concept relates only to the frequency of recognition by IgE antibodies, and it is not related to the severity of the clinical manifestations of an allergic reaction. Clinical reactions may be similar whether they are triggered by major or minor allergens. The allergenicity of each single protein is due to a number of molecular immunoreactive structures, i.e. the IgE-binding epitopes that are widespread within the protein molecule. Similar to allergens, patients allergic to the protein do not recognize all epitopes; some are immunodominant while others are only recognized by a few patients. Thus, there is room for a tremendous variation between allergic individuals with regard to the precise targets of their allergic responses.

Depending on their structure, two kinds of epitopes are described. Some are termed conformational because their

allergenicity is associated with the secondary and tertiary structure of the protein. Once the protein is denatured or hydrolyzed, conformational epitopes are modified or destroyed. Other epitopes are sequential or linear because they are formed by a sequence of amino acid residues on one peptide chain of the protein. It has recently been shown that some epitopes may have a particular clinical significance depending on their structure and location within the molecule. In children with persistent food allergy, i.e. to milk and to peanut, specific IgE binding epitopes are located in hydrophobic parts of the allergen molecules. They are buried within the tertiary structure of the protein where they are not accessible. Those short linear peptide fragments are unmasked when the protein is degraded and thus become bioavailable. Stable and resistant to proteolysis, they may remain present and immunoreactive in hydrolysates where they may also interact and reassociate in agglomerates. As compared to the native proteins, hydrolysates may thus retain a high allergenic potential of major clinical importance.

In a given allergenic food (or pollen as well) there are therefore many allergens (i.e. proteins) and in each allergen there are many allergenic structures (i.e. epitopes) that may quantitatively and qualitatively vary. The allergic individuals do not recognize all allergens and epitopes which are not always bioavailable because they may interact with other food components, environmental factors and processing. All those structures are involved in the interaction with IgE antibodies and responsible for allergenicity. The situation becomes even more complex due to the possible allergic cross-

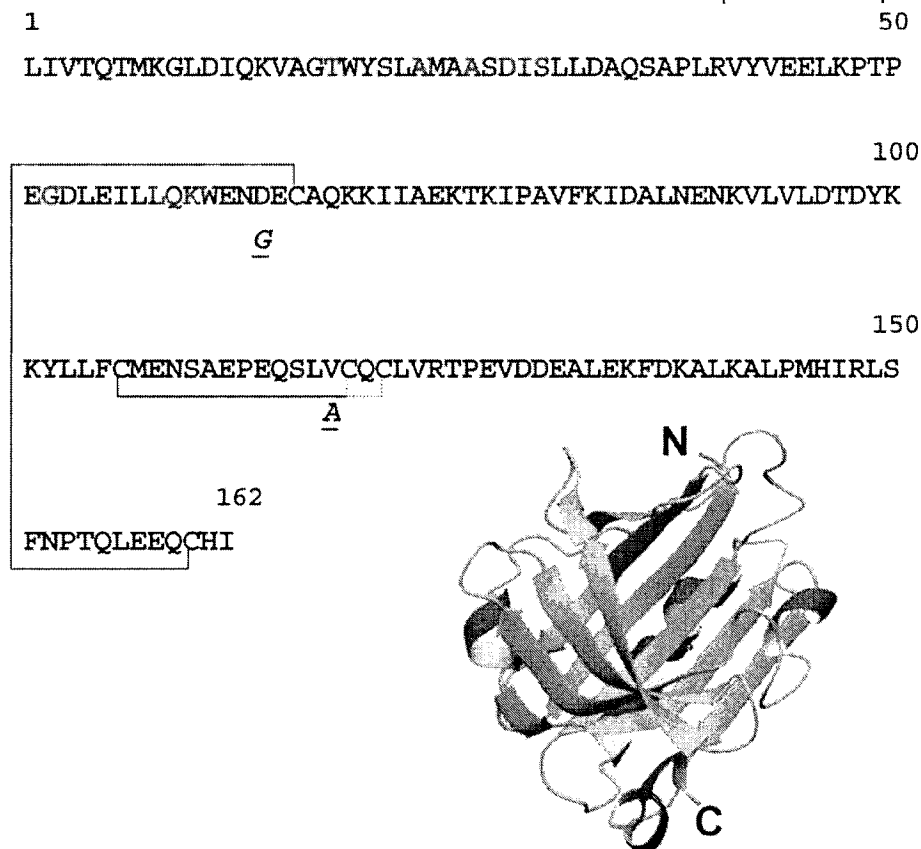


Fig. (4). 2-Dimensional and 3-D structure of cow's milk β -lactoglobulin.

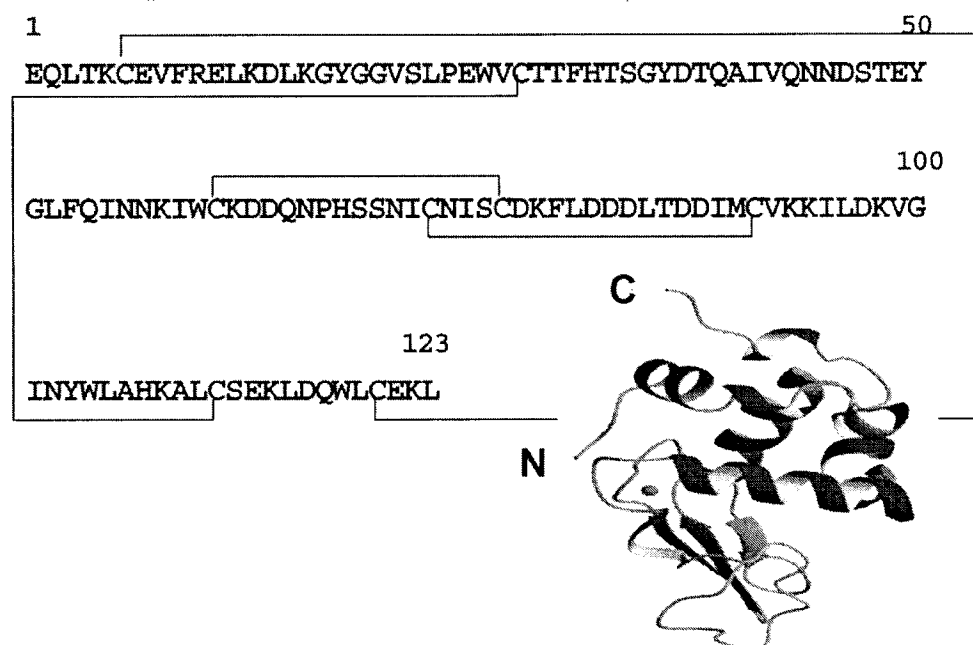


Fig. (5). 2-D and 3-D structure of cow's milk α -lactalbumin.

reactions that exist between foods or between foods and pollen. As an example, frequent cross-reactions are observed between birch pollen and hazelnut, apple and more generally fruits of the *Rosaceae* family. Cross-reactions are also observed between pollen of *Compositae* (mugwort) and celery. Also important are foods that cross-react with latex, e.g. chestnut, walnut, kiwi, banana, and avocado. Cross-reactions like those mentioned above are due to the presence of proteins that share similar epitopes.

Furthermore, in no case is the available evidence sufficient to establish an intake threshold below which allergic reactions are not triggered, or to predict reliably the effect of food processing on allergenic potential [139].

Milk allergens are a good example of the diversity and complexity of the allergen repertoire of a food and of the co-existence of conformational labile epitopes and linear stable epitopes and to the difficulty to assess whether any bioactive protein or peptide derived thereof will likely or unlikely be allergenic and present a major health concern.

Milk is one of the very common and widespread allergens that first affect atopic children in their early life. It is an allergenic food for most of the populations regardless of their geographic origin. Two percent appears to be a general mean figure for the prevalence of milk allergy in the general population of children. In unselected adults the prevalence among the general population has been reported to be around 1% [140].

As for other food allergens there are few data on thresholds of eliciting doses obtained through well-documented clinical studies. Those studies are rare and biased because testing known highly reactive patients is risky and might even be considered unethical due to the severity of clinical manifestations that may occur. However indirect indications on such threshold doses have indirectly been obtained from reports of severe adverse reactions, including anaphylactic

shocks that occurred after ingestion of minute amounts of dairy products. In addition, such adverse reactions have also been reported after ingestion of foods containing "hidden" cow's milk proteins in which the amounts of contaminating cow's milk proteins have been quantified afterwards. Such protein concentrations ranged from 0.5 to 50 ng/mL and reactions often reportedly occurred at about 5 ng/mL [141, 142, 143]. A statistical approach to determine threshold levels from published data in the literature and then to derive an "acceptable intake" for a given proportion of an allergic population (e.g. 90%), using a paradigm similar to that used in classical toxicology has been proposed. Up to now, the recorded/calculated data show that a substantial part of the population reacts to very low (in the range of micrograms) amounts of allergens, similar as those reported in cases of adverse reactions [144].

In cow's milk allergy, the main characteristic that should be underlined is the multiplicity and diversity of proteins that are involved. As shown in Fig. (6), polysensitization to several proteins most often occurs and all milk proteins appear to be potential allergens [145].

Studies on large populations of allergic patients showed that most of the patients are sensitized to several proteins including β -lactoglobulin (Bos d 5), whole casein fraction (Bos d 8), α -lactalbumin (Bos d 4), bovine serum albumin (Bos d 6), lactoferrin and immunoglobulins (Bos d 7). However, a great variability is observed in IgE-response (Fig. (7)). Caseins and β -lactoglobulin (BLG), as well as α -lactalbumin, are major allergens but proteins present in very low quantities, such as bovine serum albumin, immunoglobulins, and especially lactoferrin, also appear to be of great importance, since 35-50% of patients are sensitized to those proteins and sometimes to those proteins only. It is worth noting that in the last years, sensitivity to casein vs. whey proteins has increased in terms of both frequency and intensity of IgE response (for review see [146]).

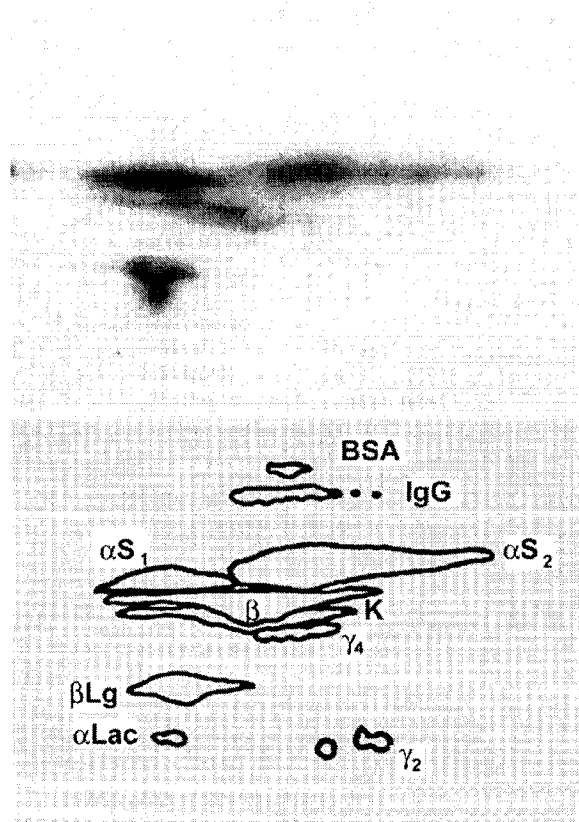


Fig. (6). Proteomic analysis of cow's milk allergens. 2-D electrophoresis combining isoelectric focusing under denaturing conditions and SDS-PAGE, applied to separate a complex protein mixture by both charge and molecular mass. The figure shows the allergenic bands recognized by IgE after the western blot performed with a milk-allergic patient serum. BSA: Bovine Serum Albumin; αS_1 , αS_2 , β , κ , γ_1 , γ_2 : different fractions of casein; βLg : β -lactoglobulin; αLac : α -lactalbumin; IgG: bovine immunoglobulin G.

It appears that three-dimensional structure is an important feature in cow's milk proteins allergenicity, but besides conformational epitopes, IgE binding studies also show the presence of sequential epitopes. These linear epitopes are peptides of various size depending on the method used to isolate and purify them, but peptides as short as ca. 12-14 amino acid residues (i.e. about 1500 Da molecular weight) have been demonstrated to account for a significant part of the allergenicity of the whole molecule in some patients. IgE binding epitopes are widely spread all along the β -lactoglobulin and α -lactalbumin molecules as evidenced by using tryptic and synthetic peptides or overlapping peptides. The best recognized peptides, by more than 90% of the patients, are BLG fragments (41-60), (102-124) and (149-162), each of them accounting for 10 to 15% of whole BLG immunoreactivity [147]. In addition the C-terminal fragment (149-162) was shown to encompass a functionally bivalent allergenic epitope, able to provoke the degranulation of mast cells and histamine release in rats sensitized to BLG [148].

Haddad *et al.*, have shown that specific IgE from patients with cow's milk allergy recognized enzymatic digestion products of BLG by pepsin or pepsin + trypsin (in all the patients), and that the recognition of peptides was even better than that of the intact molecule in 40% of the patients. They concluded that the digestive processes unmask new allergenic epitopes [149]. In allergic patients to α -lactalbumin, IgE binding to native and to large peptides confirmed the importance of conformational epitopes. However, in several patients, reduced and denatured peptides exhibited a similar or a higher IgE-binding capacity than the native corresponding fragment, suggesting the existence of sequential epitopes exposed through protein denaturation. Moreover, IgE-binding sequences are also located in strongly hydrophobic regions of the α -lactalbumin molecule where antigenicity is very unlikely to be predicted, and/or within parts of the molecule having a very high sequence homology with human α -lactalbumin [150].

Jarvinen *et al.*, using overlapping decapeptides, have identified numerous IgE- and IgG-binding epitopes of β -lactoglobulin and α -lactalbumin and they have correlated the presence of IgE to short linear epitopes with persistent (vs. transient) milk allergy [151]. Adel-Patient *et al.*, using a mouse model of milk allergy, have evidenced the impact of the conformation of the allergen on the nature of the clinical manifestations occurring during the allergic reaction to BLG. Mice sensitized to native BLG were challenged either by native or by denatured BLG. In the last case only linear epitopes were present and immediately bioavailable whereas conformational epitopes were present on native BLG. Depending on the structure of the allergen, two alternative mechanisms of mast cell activation, of different intensity, may be triggered with consequences on both the early and the late phase of the reaction. They specifically involved either peptido-leukotrienes or prostaglandin D2 in case of challenge by native or denatured BLG respectively [152].

Caseins are often considered poorly immunogenic because of their flexible, non-compact structure. They are also very susceptible to enzymatic degradation by proteases and carboxypeptidases. However they are very potent milk allergens and numerous antigenic/allergenic peptides have been evidenced among their hydrolysis-derived fragments. Table 5 shows the capacity of β -casein-derived peptides to bind serum IgE from cow's milk allergic human patients, IgG of non-allergic humans and IgG of hyperimmunized animals. It clearly indicates that short peptides may still comprise IgE binding epitope (e.g. « allergenic epitopes ») and that important differences may occur between IgE and IgG binding epitopes [146].

Most allergic patients to whole casein fraction are sensitized to each of the 4 caseins. This probably results from a co-sensitization to the different casein components after disruption of the casein micelles during the digestive process. However polysensitization also appeared to be due to cross-sensitization mechanisms and involved the acidic peptide sequences containing the cluster of phosphoserine residues: f(13-21) in β -casein, f(62-70) in α_1 -casein, and f(7-12) + f(55-60) in α_2 -casein variant A, which is the only conserved region (Fig. (8)) [153].

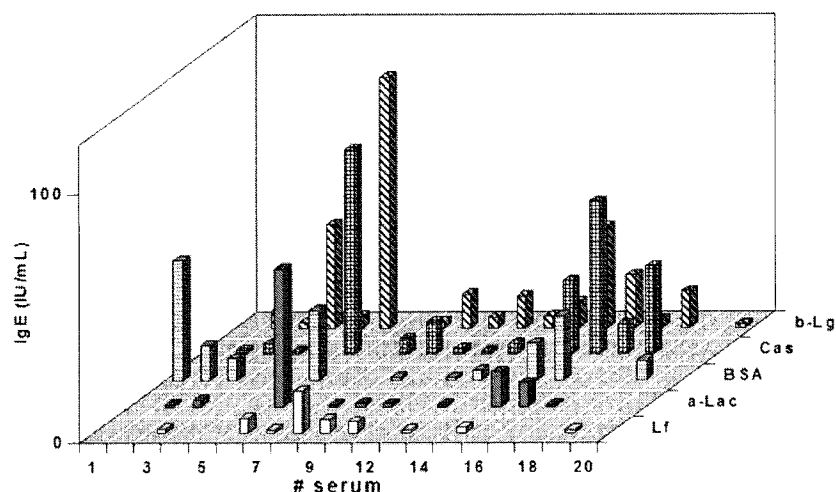


Fig. (7). Enzyme Allergo Sorbent Test determination of specific IgE to 5 milk proteins (20 patients) [146].

Table 5. Immunoreactivity of Different Classes of Anti-Bovine- β -Casein Antibodies Against Different Peptide Fragments Produced During Hydrolysis of β -Casein

β -Casein-Derived Peptide	Rabbit IgG	Human IgG	Human IgE
1-25	+	-	+
1-60	+	-	+
26-60	+	-	-
61-93	+	-	-
29-93	+	-	+
94-102	+	-	-
103-109	+	-	-
110-144	+	-	+
132-144	-	-	+
157-185	+	+	+
186-209	-	+	+

Those sequences may play an important part in allergenicity and cross-reactivity of the 3 calcium-sensitive caseins, especially since they have been described as being resistant to digestive degradation [154]. Furthermore it is worth noting that some of the major epitopes already characterized on α -caseins are continuous epitopes that have also been located in hydrophobic regions of the molecule, where they are not accessible to antibodies unless the casein is denatured or degraded, as for instance by enzymatic proteolysis during digestion [155, 156]. In addition, Chatchatee *et al.* have observed that 67 and 100% of patients over 9 years of age with persistent allergy specifically recognized sequences (69-78) and (173-194) respectively while none of the children less than 3 years old, who are likely to outgrow cow's milk allergy, had specific IgE to those fragments. In addition, sequence (69-78) was not found to be an IgG-binding epitope in any group. Such relationship between sensitization to particular short peptide fragments of cow's milk proteins and natural history of cow's milk allergy is a relevant topic in clinical practice [156].

Several T cell epitopes have also been characterized and sequence (101-112) appears to be the core sequence comprised in the peptides, which most frequently interact with HLA molecules in the immunoregulation of T cell responses to β -lactoglobulin [157].

These considerations on the structure and properties of cow's milk proteins and on the structure and location of their IgE-binding epitopes, particularly the evidence of existence and importance of short linear epitopes, have implications on the incidence of technological/physiological processing on milk allergenicity.

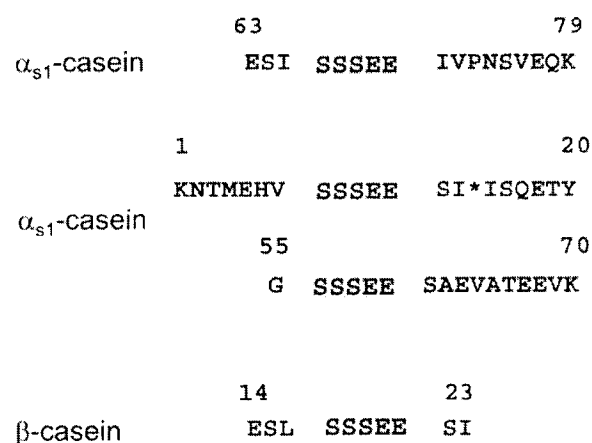


Fig. (8). Major sites of phosphorylation in caseins [153].

Effects of Processing/Digestion on Allergenicity

Heat processing has no definite or unequivocal impact on food proteins and particularly on cow's milk proteins allergenicity. Whole casein fraction is thermo-stable while β -lactoglobulin is thermo-labile but it may be protected through interaction with casein. Therefore the results depend on temperature and time of heating but also on possible in-

teractions within the food matrix. It must be emphasized that heat denaturation which leads to the loss of organized protein structures does not always result in a decreased allergenic potential. On the contrary, formation of aggregates may increase the allergenicity of the heated product. When the treatment results in a decrease of the allergenicity, it is always limited [158]. As examples, boiling of milk for a few minutes (2, 5 or 10 min) results either in no difference or in a reduction of about 50-66% of the positive allergic reactions as compared to raw milk; similar observations have been reported with raw vs. pasteurized or homogenized and pasteurized milk [159].

The route of sensitization involved in the pathogenesis of food allergy is a point of much discussion. Gastro-intestinal sensitization is likely for many food allergens for which resistance to digestion has been considered to be a prerequisite. This has led to resistance to pepsinolysis being used in a predictive mode as part of the allergenic risk assessment process for novel proteins destined for food use [160]. Later studies, however, have indicated that the relationship between resistance to digestion and allergenicity may be less clear cut than it was originally thought [161]. Recent studies on food allergens provided evidence that peptides resulting from an extensive digestion still harbor IgE-binding and/or T-cell-activating capacity [162, 163].

It is generally accepted that the hydrolysis of milk proteins considerably reduces their allergenicity and protein hydrolysates are currently used in the so-called 'hypoallergenic' formulae. However, as previously mentioned, protein breakdown products as short as 12-15 amino acid residues have been shown to still be allergenic and several studies have shown that specific IgE from patients with milk allergy recognized enzymatic digestion products of whey proteins or of the whole casein fraction and that the recognition of peptides may even be better than that of the intact molecule. Several studies have reported conflicting results on the allergenic potential of hydrolyzed formulae, depending on the nature of the proteins (e.g. whey proteins or caseins), on the enzymes used and on the degree of hydrolysis. The incidences of reported adverse effects on babies fed partially or extensively hydrolyzed milk (either casein or whey) formulae range around 45-65% and 15% respectively [164-166]. Where partially hydrolyzed formulae are concerned, allergic reactions may be due to the presence of some residual native protein or of large fragments derived therefrom. Where extensively hydrolyzed formulae are concerned, when no protein or large fragments are present, the allergic reaction may be triggered by short peptidic fragments comprising IgE-binding epitopes, such as those described above, that are released during hydrolysis.

Similar findings were obtained from the recent EU funded project "The effect of gastrointestinal digestion on the allergenicity of foods" (Allergest). *In vitro* digestion tests of Ara h1, which is a major peanut allergen, were performed with pepsin and then with trypsin plus chymotrypsin. These digestions led to a rapid and extensive degradation of the native protein, resulting in the formation of low molecular weight peptides with a M_r of less than 3,500 Da as assessed by SDS PAGE. Although the intact protein and large fragments therefrom could not be detected, no loss of allergenic-

ity was observed following hydrolysis. All the hydrolysates retained the same capacity as native Ara h1 i) to bind IgE of peanut allergic patients, ii) to degranulate sensitized basophils from those allergic patients and to release the histamine they contained, iii) to provoke an antigen-specific lymphocyte proliferation and cytokine production. In summary all the allergenic potential of the native protein was conserved in pepsin and pepsin+trypsin+chymotrypsin digests despite all the protein being hydrolyzed into small peptide fragments [167].

The risk of creating or unmasking new immunoreactive structures hitherto unseen or not bioavailable, or of over-exposure to already reactive substances may result from new food-production and processing technologies and from new dietary habits. In particular the increasing use of functional foods/food ingredients and/or of nutraceuticals derived from foods with health claims may require a safety evaluation before those novel foods/food ingredients being authorized to be placed on the market. The question is therefore: How to assess the allergenicity of such a protein or peptide fragment derived therefrom to be developed for its bioactive properties and to be used, for example, as a functional food?

Assessment of Allergenicity of a "Novel" Protein or Peptide

The following strategy has been developed and recommended by scientific committees [168, 169] for assessing the allergenicity of proteins. It may indeed be relevant for assessing the allergenicity of large peptide fragments but is not applicable for very short synthetic or highly purified peptides (i.e. 2-4 amino acid residues) as many bioactive peptides are. The object of investigation is then the source material or large intermediate breakdown fragments that may remain in trace amounts in the finished product (i.e. food-derived whole hydrolysate or hydrolysate fractions) but not the bioactive material itself.

The source of the protein must be considered carefully to make clear whether or not it is a common food allergen, then an integrated, stepwise, case-by-case approach is recommended. Given the lack of complete predictability of allergenicity a 'weight of evidence approach' aims to obtain a cumulative body of information from different sources which minimizes any uncertainty with regard to the allergenicity of the protein in question [168, 169].

Whatever the source of the protein of interest is, a first step in the assessment should be a search for sequence homologies and/or structural similarities between the protein and known allergens of different origins (e.g. food proteins, pollens). Identification of potential linear IgE binding epitopes should be conducted by a search for homologous peptidic fragments in the amino acid sequence of the protein. The number of contiguous identical or chemically similar amino acid residues used in the search setting should be based on a scientifically justified rationale in order to minimize the potential for false negative or false positive results. To reduce the uncertainty of the conclusions that may be drawn from the search of sequence homology alone, databases may be used which include information on the three dimensional structure and function of known allergens and on structural/chemical characteristics of protein families that

include a high proportion of allergens. A second step for assessing the potential that exposure to the (novel) proteins might elicit an allergic reaction in individuals already sensitized, is based on *in vitro* tests that measure the capacity of specific IgE from serum of allergic patients to bind the test protein(s).

If the protein source is considered allergenic, but no sequence homology of the (novel) protein to any known allergen is demonstrated, specific serum screening of the (novel) protein should then be undertaken with appropriate sera from patients allergic to the source material using relevant validated immunochemical tests. If a positive IgE response occurs, the (novel) protein may then be considered very likely to be allergenic. If no IgE binding is observed, the protein should undergo pepsin resistance tests and additional testing as outlined below.

If the source is not known to be allergenic but if there are consistent indications of sequence homology to a known allergen, specific serum screening should be conducted with sera from patients sensitized to this allergen in order to confirm or exclude an IgE cross-reactivity between the (novel) protein and the known allergen. The results of this screening may be interpreted as outlined above [168, 169]. The additional tests that should be performed may include the following:

Pepsin Resistance Test

Stability to digestion by proteolytic enzymes has long been considered a characteristic of allergenic proteins. Although it has now been established that no absolute correlation exists [161], the resistance of proteins to pepsin digestion is still proposed as an additional criterion to be considered in an overall risk assessment. In the case that a rapid and extensive degradation of a protein in the presence of pepsin is not confirmed under appropriate conditions, further analysis should be conducted to determine the likelihood of the (novel) protein being allergenic. It should also be useful to compare intact, pepsin digested and heat denatured proteins for IgE binding. This test may seem not to be relevant in the case where the finished product consists in extensively hydrolyzed preparations. However, as a standardized test, it may give useful indications on the stability of the source protein and therefore on the possibility that residual amounts remain in the hydrolysate that will be used as food-derived (functional) product.

Targeted Serum Screening

As proposed in the 2001 FAO/WHO expert consultation [170] targeted serum screening aims to assess the capacity of the (novel) protein to bind to IgE in sera of individuals with clinically-validated allergic responses to categories of foods broadly related to the source.

Specific (as well as targeted) serum screening requires a sufficient number and sufficient volumes of relevant sera from allergic humans. These might not always be available either because the allergy is not frequent or for other reasons. Therefore the use of existing models and the development and validation of new alternative models that can substitute for and/or complement the use of human biological material for evidence of cross reactivity and elicitation potency is

certainly a promising approach. These approaches may include the search for T-cell epitopes, structural motifs, *in vitro* cell based assays using animal or humanized-animal immune cells, etc. They may also include appropriate *in vivo* animal models.

Animal models are certainly also useful tools for the assessment of the sensitizing potential of (novel) proteins, i.e. their capacity to induce an allergic immune response with the synthesis of specific IgE in individuals that have never been exposed to those proteins/derived products nor to proteins that cross react with them. Many animal models are being developed using different routes and modes of administration, once fully validated, their use may increase the body of evidence to support a conclusion on non- or low allergenicity.

What has been outlined above is a general scheme that should be performed on a case-by-case basis. It should be noted that the preliminary step on deciding whether or not the source of the (novel) bioactive protein is a common food allergen is not as simple as it may appear. Many foods may be allergenic; some of them may have given rise to anecdotal case reports whereas others may be responsible for numerous/severe allergic reactions. Not all allergenic foods are equal but no extensive lists of scientific, objective criteria, with their weighting and rank of importance, have been defined so far for identifying allergic foods of public health importance. Such lists should take into account some relevant criteria such as:

- Frequency or prevalence either worldwide or in some limited geographic areas with particularities in the dietary habits of the population,
- Severity and persistence of the allergic reactions to those foods
- Level of intake and exposure, e.g. average daily intake as a food but also as food-derived products that may be used in numerous recipes of fabricated foods and present as (hidden) ingredient in finished products,
- Stability through processing and digestion

Several lists of most common allergenic foods have been proposed such as the so-called "Big Eight" and more recently by the European Commission for labeling purposes (Table 6) [171]. These lists are supposed to take into account around 80% of all/global food allergy although differences may be observed depending on socio-cultural and geographic conditions.

The EU list has been established to provide comprehensive and reliable information to the allergic consumer on the unexpected presence of an allergenic food ingredient to which they are sensitized in the food they purchase. This means that other common allergenic foods such as fruits of the latex group (kiwi, banana, etc.), fruits of the *Rosaceae* family (apples, pears, prunes) or vegetables that are common allergens for adults but that can most often be recognized as such are not included in the list.

Each category of foods not only concerns the native food but products derived therefrom, which means that any derived product obtained from those foods using different types of processing protocols are *a priori* considered allergenic

Table 6. List of Common Allergenic Foods/Food Ingredients Mentioned in Annex 3a to the Directive 2003/89/EC for labeling Purposes

Cereals containing gluten (i.e. wheat, rye, barley, oats, spelt, kamut or their hybridized strains) and products thereof
Crustaceans and products thereof
Eggs and products thereof
Fish and products thereof
Peanuts and products thereof
Soybeans and products thereof
Milk and products thereof (including lactose)
Nuts i.e. Almond (<i>Amygdalus communis</i> L.), Hazelnut (<i>Corylus avellana</i>), Walnut (<i>Juglans regia</i>), Cashew (<i>Anacardium occidentale</i>), Pecan nut (<i>Carya illinoensis</i> (Wangenh.) K. Koch), Brazil nut (<i>Bertholletia excelsa</i>), Pistachio nut (<i>Pistacia vera</i>), Macadamia nut and Queensland nut (<i>Macadamia ternifolia</i>) and products thereof
Celery and products thereof
Mustard and products thereof
Sesame seeds and products thereof
Sulfur dioxide and sulfites at conc. of more than 10 mg/kg or 10 mg/L expressed as SO ₂ .

and should be labeled with reference to the source. There might be specific derivatives of these known ingredients that do not trigger an allergic reaction, but this would need to be evaluated on a case-by-case basis (see below). This includes whole hydrolysates and isolated peptides that have been produced from allergenic foods such as milk or eggs and incorporated in other foods or finished products as functional/bioactive ingredients.

Based on what is known on the structure of some milk and peanut allergen epitopes such as β -lactoglobulin and Ara h1 and on the limited impact of proteolysis and digestion on the allergenicity of those proteins, it is likely that food processing and particularly hydrolysis may not fully or even partially destroy the allergenicity of a protein known to be a common allergen. It may not even reduce it significantly as to protect a vast majority of allergic patients to this protein or to the source of this protein. Since allergenicity cannot be definitely associated to exposure to a well-defined, constant, universal threshold level below which the protein would not elicit an allergic reaction, the monitoring of the residual level of the native protein in hydrolysates is not a sufficient indication of any relevant decrease of the allergenic potential. In addition within the context of labeling regulations, risk assessors do not consider that the lack of reported case of allergic reaction to a product derived from an allergic food is a demonstration of the absence of allergenicity. This has recently been shown to be the case when producers of wheat gluten and soy hydrolysates applied for an exemption of labeling for the use these hydrolysates as food ingredients. Wheat and soy are on the list of most common allergenic foods and it is fully justified to implicate wheat gluten and soy-derived products, namely hydrolysates, for their potential to induce allergy.

Cases of anaphylaxis due to wheat isolates in food were reported [172]. Indeed even enzymatic hydrolysis of wheat gluten is not a sign of lack of allergenicity [173, 174].

Regarding acid hydrolyzed vegetable proteins from soy, it has been observed that hydrolysates may contain low levels of known and novel allergenic proteins and peptides. It is not known at which levels of intake soy protein based acid hydrolyzed proteins would cause allergic reactions in soy allergic individuals.

In both cases the European Commission and the European Food Safety Authority (EFSA) have recommended to conduct comprehensive studies including appropriate clinical studies applying best clinical and laboratory practice [175, 176].

Very few comprehensive well-conducted clinical studies are available to give appropriate and sufficient evidence of non-allergenicity of hydrolysates (or of purified compounds isolated therefrom) that are produced from allergenic intact food proteins. Another point to consider is whether such hydrolysates may sensitize consumers after their long-term consumption. This has been even less investigated. Some approaches have been performed within the EU funded projects "Caseinophosphopeptides (CPPs) - nutraceutical/functional food ingredients for food and pharmaceutical applications" (FICAPPs) and "Hypotensive peptides from milk proteins" (HTMProt) in order to assess the allergenicity of milk protein hydrolysates.

In the context of the FICAPPs project, analysis of the immune response (e.g. induction of specific IgE and IgGs production) to oral consumption of CPPs was monitored in human volunteers, who were not allergic to milk. Those volunteers consumed CPPs derived from cow's milk caseins (i.e. α_{s1} -casein, α_{s2} -casein, β -casein and κ -casein) as functional food ingredients in their diet. Volunteer sera were tested for specific anti-CPP-IgE, -IgG₁, -IgG₂, -IgG₃ and -IgG₄ on week 0, 1, 2, 3, 4, 5 and 6 of the feeding trial³.

³ Bernard H *et al.*; unpublished results

A great heterogeneity in intensity and specificity of the "basal" human IgG response to CPPs was observed before the beginning of the feeding trial (week 0) but no evolution of the IgG (e.g. IgG₁, IgG₂, IgG₃ and IgG₄) response either in intensity or in specificity was observed in all the volunteers during the feeding trial and up to two weeks after the consumption of CPPs ceased. IgG₄ response to any of the CPP appears to be the most variable and β -casein f(1-25) appears to induce the most variable response irregardless of the IgG sub class. The observed inter individual variations may be due to human genetic variability or to different dietary habits occurring in the different volunteers. No anti-CPP-IgE were detected either before or after the feeding trial in any volunteer, which indicated that CPPs do not induce an allergic sensitization in consumers who are not originally allergic to milk. Long-term ingestion of CPPs did not induce any specific anti-phosphopeptide IgG-response, which indicated that CPPs are not immunogenic.

As a conclusion, no immunological effect of CPPs, at least on the antibody response, was observed on human volunteers during a long term feeding trial. However no definite opinion can be expressed in terms of risk assessment of the allergic potential of CPPs because it cannot be excluded that CPP preparations may elicit a reaction in some patients who are allergic to milk casein. Appropriate information (e.g. through labeling) should therefore be given that CPP preparations and CPP fortified foods are not suitable for individuals who are allergic to milk proteins.

The HTMProt project dealt with milk protein hydrolysates with potential hypotensive activity. The pharmacological activity quantified was the potential for inhibition of angiotensin converting enzyme (ACE) activity and the most efficient hydrolysate preparations, were tested on human volunteers during well-controlled and designed clinical trials. The hydrolysates produced using authorized food grade proteases only contained peptides but no residual intact milk proteins.

Assessment of the allergenicity of the preparations was performed by Enzyme Allergo Sorbent Assay (EAST) and EAST inhibition using the sera of allergic patients to milk proteins and β -lactoglobulin. It was observed that the hydrolysates exhibit a strongly decreased IgE binding capacity but still had a significant residual allergenicity. These potentially hypotensive hydrolyzed milk protein preparations are thus not suitable for consumption by milk allergic patients.

As was the case for CPPs, analysis of (adverse) effects on the immune response in humans fed the potentially hypotensive peptides and particularly the induction of any immune response by a long-term administration of the product was assessed by measuring serum-specific anti-milk-protein-antibodies in non-allergic human volunteers, before, during and after the feeding trial. The feeding trial, as expected, did not sensitize the volunteers to milk proteins⁴.

In conclusion, no or very weak effects of long-term feeding on the immune response and particularly on the induction of a specific antibody response were evidenced with the

different potentially hypotensive milk protein hydrolysate preparations. However, again as with the CPP study, these milk-protein-derived preparations, although they were produced by hydrolysis, are definitely not appropriate for consumers who are allergic (i.e. already sensitized) to cow's milk proteins as it is possible that they may elicit an allergic reaction in this population grouping.

CONCLUSIONS

Cytotoxicity

There are numerous proteins and peptides of plant and animal origin that may be ingested as a normal or functional/novel food and exhibit a certain cytotoxic potential in the human system.

It is quite clear that this can result in limitations to the consumption of such foods. Therefore these effects have to be examined with regard to dose-dependency and the underlying mode of action. Only if the adverse effect is absent or negligible the ascribed health claim of the respective compound or product should be postulated without restrictions.

It is worth noting that many studies report on a cytotoxic potential of bioactive peptides and proteins that is exclusively or at least to a higher extent observed in experiments with malignant cells. In fact, many plant proteins, especially lectins and gamma-thionins (defensins), and peptides have been ascribed a beneficial potential for cancer therapy or prophylaxis. Besides some milk proteins and peptides also seem to have a certain effect on cancer cells. Especially the whey protein lactoferrin and the derived peptide lactoferricin or fragments thereof are effective as well as casein-derived peptides that also exhibit antimicrobial properties (casecidins).

A strongly protective effect of whey protein concentrate on dimethylhydrazine-induced colon cancer in the rat has been reported [177]. Moreover, the whey protein α -lactalbumin seems to have anticancer potential towards a variety of cells including malignant cells of the gut epithelium. Human α -lactalbumin has been extensively studied for its beneficial properties. This has led to the development of human α -lactalbumin made lethal to tumor cells (HAMLET). HAMLET induces apoptosis in tumor cells but leaves normal differentiated cells unaffected. After partial unfolding and binding to oleic acid, α -lactalbumin forms the HAMLET complex, which enters tumor cells and freezes their meta-

bolic machinery. The cells proceed to fragment their DNA, and they disintegrate with apoptosis-like characteristics. HAMLET kills a wide range of malignant cells *in vitro* [178].

In general, cytotoxicity caused by food proteins and peptides seems to play a crucial role in the regulation of human bodily functions and development. Further research needs to be performed in order to elucidate and understand how - and to what extent - the original or added/processed food components influence the development of cancer *in vivo*.

The development and application of *in vitro* assays as screening tools can help estimate potential cytotoxicity prior to applying such compounds in animal or human studies.

⁴ Bernard H et al.: unpublished results

This helps to minimize cost-intensive human studies as well as the use of laboratory animals [179].

The cell culture system chosen for cytotoxicity assessment is crucial for a reasonable evaluation of the compounds tested. While many cells are applicable in screening tests, the cells applied for a more precise experiment should be closely related to the expected target cells *in vivo*. Peptides have been shown to enter the circulation for example by paracellular transport routes (tight junctions) without prior degradation and thus can affect different target cells in the human system [180].

It is worth noting that a cytotoxic response found for a certain peptide may be overestimated unless it is evaluated in relation to the cytotoxic potential of the original protein the peptide had been released from. Based on results from own studies, we therefore suggest that it is essential not to overestimate the cytotoxicity of peptides derived from normal food proteins. These intact food proteins themselves, e.g. β -lactoglobulin and Na-caseinate could also inhibit the metabolic activity (WST-1 assay) of different cell types *in vitro*.⁵ Peptides derived from these common food ingredients had comparable effects towards the cell types investigated. Consequently, the relative cytotoxicity index (RCI) was calculated, as a quotient of the peptide effect and the effect of the original protein. One disadvantage of this "RCI-method" is that it is not applicable to (synthetic) peptides whose sequence is not encrypted in food proteins.

To date, there is a large range of cytochemical assay systems the scientist potentially available when assessing cytotoxicity. Some of these assays can be used to screen a battery of compounds and concentrations for unspecific cytotoxic events. In the field of biological evaluation of medical devices, the international standard DIN EN ISO 10993-5 describes the requirements for cytotoxicity assays and cell culture model systems to be used for such purposes [181]. Such standards which hold a certain legal character help to increase acceptance of these cytochemical methods.

Intestinal cell culture systems have received increased attention as models for toxicological studies, since the intestine is among the primary sites of exposure to ingested bioactive substances and toxicants. One main advantage of this assay type is the possibility to carry it out repeatedly over a longer period as it is non-destructive.

Many studies have shown that cytotoxic compounds can disturb normal epithelial function in the gut, also affecting the paracellular transport route [180]. The methods to determine epithelial integrity and functionality (TEER, mannitol/fluorescein) have shown close correlation to results obtained with the LDH leakage assay. However, the TEER value is affected at lower concentrations of a cytotoxic compound than that required for LDH release through membrane lesions. This means that TEER measurements can reveal adverse effects on human gut epithelial functions even if the classical LDH assay would indicate the absence of cytotoxicity [182, 183]. This fact underlines the particular importance of epithelial cell culture models for a holistic cytotoxicity assessment of proteins and peptides.

Allergenicity

The constituents that are responsible for allergenicity of foods as well as of pollens are proteins. Some protein breakdown products, i.e. peptide fragments, may conserve part of the allergenicity of the native protein and thus can also be considered as allergens.

Allergenicity is not an intrinsic, fully predictable property of a given protein but is a biological activity requiring an interaction with individuals with a pre-disposed genetic background. Allergenicity therefore depends upon the genetic diversity and variability in atopic humans. Given this lack of complete predictability it is necessary to obtain, from several steps in the risk assessment process, a cumulative body of evidence that minimizes any uncertainty with regard to the protein in question. Whether the bioactive protein or derived peptide(s) comes from a known allergenic source or not is obviously of major importance in the assessment process.

A list of the most common allergenic foods has been established for labeling purpose. This list which may evolve depending on changes in dietary habits, food-processing practices etc. includes allergenic foods and all associated or derived products including hydrolysates. However, the way processing may alter (e.g. decrease or increase) the allergenic potential cannot be predicted. In general, hydrolysates do not only contain very short bioactive peptides but also residual amounts of protein or large breakdown fragments. Current scientific evidence is insufficient to establish an intake threshold for any of these ingredients, i.e., below which an allergic reaction may not occur. Furthermore, the possibility that specific well characterized derivatives of these food allergens, produced for a particular use by a well defined and controlled processing protocol, are unlikely to trigger an allergic reaction in the vast majority of allergic patients needs to be evaluated on a case-by-case basis.

In order to provide a sufficient level and quality of evidence of non-allergenicity several kinds of data are required. They include:

- History of non-allergenicity of the product based on a well documented literature review strategy
- Laboratory-based tests (e.g. *in vitro* studies, protein analysis and determinations, immunological methods, animal studies)
- Clinical studies (e.g. skin tests, double-blind placebo-controlled food challenge)

Of great importance is the fact that laboratory tests on allergic human sera/cells as well as clinical studies are performed on appropriate groups of relevant patients with a well-characterized allergy to the source protein, using good laboratory and clinical practices.

ACKNOWLEDGEMENTS

The first author would like to express his thankfulness to Prof. Dr. Hans Meisel for his professional cooperation and his continuous support concerning all research activities.

Part of the studies reported on had been carried out with financial support from the European Commission program,

⁵ Pentzien AK: unpublished results

CT98-3077, "Caseinophosphopeptides: Nutraceutical/Functional Food Ingredients for Food and Pharmaceutical Applications" and "Hypotensive Peptides from Milk Proteins" (QLK1-CT20000-00043); the present publication does not necessarily reflect the Commission's views and in no way anticipates its future policy in this area.

ABBREVIATIONS

AA	=	Amino acid(s)
ACE	=	Angiotensin-converting enzyme
BB	=	Bowman-Birk
BLG	=	β -lactoglobulin
CPP	=	Caseinophosphopeptides
DPP IV	=	Dipeptidyl aminopeptidase IV
EAST	=	Enzyme Allergo Sorbent Assay
HAMLET	=	Human α -lactalbumin made lethal to tumor cells
IC ₅₀	=	Inhibitor concentration leading to 50 % inhibition of activity
LA 70	=	Lactalbumin 70
LDH	=	Lactate dehydrogenase
NR	=	Neutral red
PI	=	Protease inhibitor
RCI	=	Relative cytotoxicity index
RIP	=	Ribosome-inactivating protein
SDS-PAGE	=	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TEER	=	Transepithelial electrical resistance
WPC	=	Whey protein concentrate

REFERENCES

References 184-186 are related articles recently published in *Current Pharmaceutical Design*.

- Bruijnzeel-Koomen C, Ortolani C, Aas K, Bindslev-Jensen C, Bjorksten B, Moneret-Vautrin D, *et al.* Adverse reactions to food. *Allergy* 1995; 50: 623-35.
- Bantel H, Engels IH, Voelter W, Schulze-Osthoff K, Wesselborg S. Mistletoe lectin activates caspase-8/FLICE independently of death receptor signaling and enhances anticancer drug-induced apoptosis. *Cancer Res* 1999; 59: 2083-90.
- Gulden M, Seibert H. *In vitro* - *in vivo* extrapolation: estimation of human serum concentrations of chemicals equivalent to cytotoxic concentrations *in vitro*. *Toxicology* 2003; 189: 211-22.
- Green DR. Apoptotic pathways: paper wraps stone blunts scissors. *Cell* 2000; 102: 1-4.
- Barrett KL, Willingham JM, Garvin AJ, Willingham MC. Advances in cytochemical methods for detection of apoptosis. *J Histochem Cytochem* 2001; 49: 821-32.
- Fotakis G, Timbrell JA. *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett* 2006; 160: 171-7.
- Decker T, Lohmann-Matthes M. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Meth* 1988; 115: 61-9.
- Dierickx PJ. Comparison of the cytotoxicity of the MEIC reference chemicals measured in protein free and in complete culture medium. *Toxicol In Vitro* 2005; 19: 859-64.
- Dierickx PJ, Scheers EM. Neutral red uptake inhibition in adhered and adhering rat hepatoma-derived Fa32 cells to predict human toxicity. *J Appl Toxicol* 2002; 22: 61-5.
- Dalla Pellegrina C, Rizzi C, Mosconi S, Zoccatelli G, Peruffo A, Chignola R. Plant lectins as carriers for oral drugs: Is wheat germ agglutinin a suitable candidate? *Toxicol Appl Pharmacol* 2005; 207: 170-8.
- Tan AS, Berridge MV. Superoxide produced by activated neutrophils efficiently reduces the tetrazolium salt, WST-1 to produce a soluble formazan: a simple colorimetric assay for measuring respiratory burst activation and for screening anti-inflammatory agents. *J Immunol Methods* 2000; 21: 59-68.
- Huhtala A, Alajuuja P, Burgalassi S, Chetoni P, Diehl H, Engelke M, *et al.* A collaborative evaluation of the cytotoxicity of two surfactants by using the human corneal epithelial cell line and the WST-1 test. *J Ocul Pharmacol Ther* 2003; 19: 11-21.
- Willingham MC. Cytochemical methods for the detection of apoptosis. *J Histochem Cytochem* 1999; 47: 1101-9.
- Huang X, Halicka HD, Traganos F, Tanaka T, Kurose A, Darzynkiewicz Z. Cytometric assessment of DNA damage in relation to cell cycle phase and apoptosis. *Cell Prolif* 2005; 38: 223-43.
- Green AM, Steinmetz ND. Monitoring apoptosis in real time. *Cancer J* 2002; 8: 82-92.
- Boyce M, Degterev A, Yuan J. Caspases: an ancient cellular sword of Damocles. *Cell Death Differ* 2004; 11: 29-37.
- Saunders PA, Cooper JA, Roodell MM, Schroeder DA, Borchert CJ, Isaacson AL, *et al.* Quantification of active caspase-3 in apoptotic cells. *Anal Biochem* 2000; 284: 114-24.
- Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980; 284: 555-6.
- Winkelmeier P, Glauner B, Lindl T. Quantification of cytotoxicity by cell-volume and cell-proliferation. *ATLA-Altern Lab Anim* 1993; 21: 269-80.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-75.
- Tamarit A, Alberola J, Galindo MJ, Navarro D. Application of a 5-bromo-2'-deoxyuridine ELISA for measuring the lymphoproliferative response to human cytomegalovirus in HIV-1-infected patients. *J Virol Methods* 2002; 105: 247-51.
- Messele T, Roos MTL, Hamann D, Koot M, Fontanet AL, Miedema F, *et al.* Nonradioactive techniques for measurement of *in vitro* T-cell proliferation: Alternatives to the [H-3]thymidine incorporation assay. *Clin Diag Lab Immun* 2000; 7: 687-92.
- Berridge MV. The Biochemical and Cellular Basis of Cell Proliferation Assays That Use Tetrazolium Salts. *Biochemica* 1996; 4: 15-9.
- Matsumoto H, Erickson R, Gum J, Yoshioka M, Gum E, Kim Y. Biosynthesis of alkaline phosphatase during differentiation of the human colon cancer cell line caco-2. *Gastroenterology* 1990; 98: 1199-207.
- Hartmann R, Meisel H. Cytochemical assessment of phosphopeptides derived from casein as potential ingredients for functional food. *Nahrung/Food* 2002; 46: 427-31.
- Okada T, Narai A, Matsunaga S, Fusetani N, Shimizu M. Assessment of the marine toxins by monitoring the integrity of human intestinal Caco-2 cell monolayers. *Toxicol In Vitro* 2000; 14: 219-26.
- Van Dyke R. Mechanisms of digestion and absorption of food. In: Sleisenger M, Fordtran J Eds, *Gastrointestinal Disease, Pathophysiology, Diagnosis, and Management*. Philadelphia, Saunders Company 1989; 1062-88.
- Konsoula R, Barile FA. Correlation of *in vitro* cytotoxicity with paracellular permeability in Caco-2 cells. *Toxicol In Vitro* 2005; 19: 675-84.
- Borenfreund E, Puerner JA. A simple quantitative procedure using monolayer culture for toxicity assays. *J Tissue Cult Meth* 1984; 9: 7-9.
- Olivier A, Van Wyk CW. Cytotoxicity assays - Neutral red and tetrazolium: a comparison. *J Dent Res* 2002; 81: B375 Sp. Iss. B.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival - application to proliferation and cyto-toxicity assays. *J Immunol Methods* 1983; 65: 55.

- [32] Yang TT, Sinai P, Kain SR. An acid phosphatase assay for quantifying the growth of adherent and nonadherent cells. *Anal Biochem* 1996; 241: 103-8.
- [33] Hartmann R, Meisel H. Caseinophosphopeptides and their cell modulating potential. *Biofactors* 2004; 21: 73-8.
- [34] Drastichova J, Svestkova E, Luskova V, Svobodova Z. Cytochemical study of carp neutrophil granulocytes after acute exposure to cadmium. *J Appl Ichthyol* 2005; 21: 215-9.
- [35] Raspotnig G, Fauler G, Jantscher A, Windischhofer W, Schachl K, Leis HJ. Colorimetric determination of cell numbers by Janus green staining. *Anal Biochem* 1999; 275: 74-83.
- [36] Asbury CL, Esposito R, Farmer C, van den Engh G. Fluorescence spectra of DNA dyes measured in a flow cytometer. *Cytometry* 1996; 24: 234-42.
- [37] Kato F, Tanaka M, Nakamura K. Rapid fluorometric assay for cell viability and cell growth using nucleic acid staining and cell lysis agents. *Toxicol In Vitro* 1999; 13: 923-9.
- [38] Martin RM, Leonhardt H, Cardoso MC. DNA labelling in living cells. *Cytometry Part A* 2005; 67A: 45-52.
- [39] Williamson P, Schlegel RA. Transbilayer phospholipids movement and the clearance of apoptotic cells. *Biochem Biophys Acta* 2002; 30: 53-63.
- [40] Jacobson MD, Wall M, Ralf MC. Programmed cell death in animal development. *Cell* 1997; 88: 347-54.
- [41] Ralf M. Cell suicide for beginners. *Nature (London)* 1998; 395: 119-22.
- [42] Otton R, Curi R. Toxicity of a mixture of fatty acids on human blood lymphocytes and leukaemia cell lines. *Toxicol in Vitro* 2005; 19: 749-55.
- [43] Garrido C, Kroemer G. Life's smile, death's grin: vital functions of apoptosis-executing proteins. *Curr Opin Cell Biol* 2004; 16: 639-46.
- [44] Nicotera P. Apoptosis and age-related disorders: role of caspase-dependent and caspase-independent pathways. *Toxicol Lett* 2002; 127: 189-95.
- [45] Maghni, K; Nicolescu, OM; Martin, JG. Suitability of cell metabolic colorimetric assays for assessment of CD4+ T cell proliferation: comparison to 5-bromo-2-deoxyuridine (BrdU) ELISA. *J Immunol Methods* 1999; 223: 185-94.
- [46] Morobu M, El-Abasy M, Na KJ, Hirota Y. Detection of mitogen-induced lymphocyte proliferation by bromodeoxyuridine (BrdU) incorporation in the chicken. *J Vet Med Sci* 2002; 64: 377-9.
- [47] Elion EA. The Ste5p scaffold. *J Cell Sci* 2001; 114: 3967-78.
- [48] Bradford M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem* 1976; 72: 248-54.
- [49] Pinto M, Robineleon S, Appay Md, Kedinger M, Triadou N, Dussaulx E, *et al.* Enterocyte-like differentiation and polarization of the human-colon carcinoma cell-line Caco-2 in culture. *Biol Cell* 1983; 47: 323-30.
- [50] Suzuki T, Hara H. Various nondigestible saccharides open a paracellular calcium transport pathway with the induction of intracellular calcium signalling in human intestinal Caco-2 cells. *J Nutr* 2004; 134: 1935-41.
- [51] Nusrat A, Brown GT, Tom J, Drake A, Bui TTT, Quan C, *et al.* Multiple protein interactions involving proposed extracellular loop domains of the tight junction protein occluding. *Mol Biol Cell* 2005; 16: 1725-34.
- [52] Fanning AS, Jameson BJ, Jesaitis LA, Anderson JM. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem* 1998; 273: 29745-53.
- [53] Konishi Y. Modulations of Food-derived Substances on Intestinal Permeability in Caco-2 Cell Monolayers. *Biosci Biotechnol Biochem* 2003; 67: 2297-9.
- [54] Alpers DH. Digestion and absorption of carbohydrates and proteins. In: Johnson LR Ed, *Physiology of the gastrointestinal tract*. New York, Raven Press 1994; Vol. 3: 1723-50.
- [55] Tobey N, Heizer W, Yeh R, Huang TI, Hoffner C. Human intestinal brush border peptidases. *Gastroenterology* 1985; 88: 913-26.
- [56] Yoshioka M, Erickson RH, Matsumoto H, Gum E, Kim YS. Expression of dipeptidyl aminopeptidase-IV during enterocytic differentiation of human colon cancer (Caco-2) cells. *Int J Cancer* 1991; 47: 916-21.
- [57] Meisel H. Biochemical properties of peptides encrypted in bovine milk proteins. *Curr Med Chem* 2005; 12: 1905-19.
- [58] Meisel H. Bioactive peptides from milk proteins: a perspective for consumers and producers. *Aust J Dairy Technol* 2001; 56: 83-92.
- [59] Bezault J, Bhimani R, Wiprovnick J, Furmanski P. Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. *Cancer Res* 1994; 54: 2310-2.
- [60] Van't Land B, Van Beek NMA, Van den Berg JJM, M'Rabet L. Lactoferrin reduces methotrexate-induced small intestinal damage, possibly through inhibition of GLP-2-mediated epithelial cell proliferation. *Dig Dis Sci* 2004; 49: 425-33.
- [61] Sakai T, Banno Y, Kato Y, Nozawa Y, Kawaguchi M. Pepsin-digested bovine lactoferrin induces apoptotic cell death with JNK/SAPK activation in oral cancer cells. *J Pharmacol Sci* 2005; 98: 41-8.
- [62] Gifford JL, Hunter HN, Vogel HJ. Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell Mol Life Sci* 2005; 62: 2588-98.
- [63] Eliassen LT, Berge G, Sveinbjornsson B, Svendsen JS, Vorland LH, Rekdal O. Evidence for a direct antitumor mechanism of action of bovine lactoferricin. *Anticancer Res* 2002; 22: 2703-10.
- [64] Nguyen LT, Schibli DJ, Vogel HJ. Structural studies and model membrane interactions of two peptides derived from bovine lactoferricin. *J Pept Sci* 2005; 11: 379-89.
- [65] Roy MK, Kuwabara Y, Hara K, Watanabe Y, Tamai Y. Peptides from the N-terminal end of bovine lactoferrin induce apoptosis in human leukemic (HL-60) cells. *J Dairy Sci* 2002; 85: 2065-74.
- [66] Schlimme E, Meisel H, Frister H. Bioactive sequences in milk proteins. In: Barth CA, Schlimme E Eds, *milk proteins*. Darmstadt, Springer 1988; 143-9.
- [67] Hata I, Higashiyama S, Otani H. Identification of a phosphopeptide in bovine alpha(s1)-casein digest as a factor influencing proliferation and immunoglobulin production in lymphocyte cultures. *J Dairy Res* 1998; 65: 569-78.
- [68] Jiang YJ, Li QZ, Yan HB, Geng LJ. Expression and bioactivity analysis of recombinant beta-CPP dimmer. *J Dairy Sci* 2004; 87: 3198-208.
- [69] Otani H, Suzuki H. Isolation and characterization of cytotoxic small peptides, alpha-caseicidins, from bovine alpha₁-casein digested with bovine trypsin. *Anim Sci J* 2003; 74: 427-35.
- [70] Matin MA, Otani H. Cytotoxic and antibacterial activities of chemically synthesized kappa -caseicin and its partial peptide fragments. *J Dairy Res* 2002; 69: 329-34.
- [71] Matin MA, Monnai M, Otani H. Isolation and characterization of a cytotoxic pentapeptide, kappa-caseicin, from bovine kappa-casein digested with bovine trypsin. *Anim Sci J* 2000; 71: 197-207.
- [72] Matin MA, Otani H. Release of cytotoxic glycopeptides from human acid casein fraction by the action of stomach proteinases. *Milchwissenschaft* 2000; 55: 6-10.
- [73] Xu MY, Sugiura Y, Nagaoka S, Kanamaru Y. IEC-6 intestinal cell death induced by bovine milk alpha-lactalbumin. *Biosci Biotechnol Biochem* 2005; 69: 1082-9.
- [74] Xu MY, Sugiura Y, Nagaoka S, Kanamaru Y. Involvement of SDS-stable higher M_r forms of bovine normal milk alpha-lactalbumin in inducing intestinal IEC-6 cell death. *Biosci Biotechnol Biochem* 2005; 69: 1189-92.
- [75] Permyakov EA, Berliner LJ. alpha-Lactalbumin: structure and function. *FEBS Lett* 2000; 473: 269-74.
- [76] Svensson M, Sabharwal H, Hakansson A, Mossberg AK, Lipniunas P, Leffler H, *et al.* Molecular characterization of alpha-lactalbumin folding variants that induce apoptosis in tumor cells. *J Biol Chem* 1999; 274: 6388-96.
- [77] Matin MA, Mizumoto G, Otani H. Cytotoxic properties of alpha-lactalbumins toward mammalian cells. *Milchwissenschaft* 2001; 56: 430-3.
- [78] Hsieh CL, Ho YC, Lai HH, Yen GC. Inhibitory effect of camosine and anserine on DNA oxidative damage induced by Fe2+, Cu2+ and H2O2 in lymphocytes. *J Food Drug Anal* 2002; 10: 47-54.
- [79] Hipkiss AR. Carnosine, a protective, anti-ageing peptide? *Int J Biochem Cell Biol* 1998; 30: 863-8.
- [80] Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H, Svanborg C. Apoptosis Induced By A Human-Milk Protein. *Proc Natl Acad Sci USA* 1995; 92: 8064-8.
- [81] Thompson LU. Potential Health Benefits And Problems Associated With Antinutrients In Foods. *Food Res Int* 1993; 26: 131-49.
- [82] Philips BJ. Development of cell culture techniques for assessment of the toxicity of plant products. *Toxicol In Vitro* 1996; 10: 69-76.

- [83] De Mejia EG, Prisecaru VI. Lectins as bioactive plant proteins: A potential in cancer treatment. *Crit Rev Food Sci Nutr* 2005; 45: 425-45.
- [84] Gibbs BF, Alli I. Characterization of a purified alpha-amylase inhibitor from white kidney beans (*phaseolus vulgaris*). *Food Res Int* 1998; 31: 217-25.
- [85] Mourey L, Pedelacq JD, Bircck C, Fabre C, Rouge P, Samama JP. Crystal structure of the arcelin-1 dimer from *phaseolus vulgaris* at 1.9-angstrom resolution. *J Biol Chem* 1998; 273: 12914-22.
- [86] Ye XY, Ng TB, Rao PF. A Bowman-Birk-type trypsin-chymotrypsin inhibitor from broad beans. *Biochem Biophys Res Comm* 2001; 289: 91-6.
- [87] Barciszewski J, Szymanski M, Haertle T. Minireview: Analysis of rape seed napin structure and potential roles of the storage protein. *J Protein Chem* 2000; 19: 249-54.
- [88] Ngai PHK, Ng TB. A napin-like polypeptide with translation-inhibitory, trypsin-inhibitory, antiproliferative and antibacterial activities from kale seeds. *J Pept Res* 2004; 64: 202-8.
- [89] Steiner R. *Geisteswissenschaft und Medizin*. Rudolf Steiner Verlag, Dornach 1985.
- [90] Steuer-Vogt MK, Bonkowsky V, Ambrosch P, Scholz M, Neiss A, Strutz J, et al. The effect of an adjuvant mistletoe treatment programme in resected head and neck cancer patients: a randomised controlled clinical trial. *Eur J Cancer* 2001; 37: 23.
- [91] Thomma BPHJ, Cammue BPA, Thevissen K. Plant defensins. *Planta* 2002; 216: 193-202.
- [92] Kagan BI, Ganz T, Lehrer RI. Defensins - A family of antimicrobial and cytotoxic peptides. *Toxicology* 1994; 87: 131-49.
- [93] Hughes P, Dennis E, Whitecross M, Llewellyn D, Gage P. The cytotoxic plant protein, beta-purothionin, forms ion channels in lipid membranes. *J Biol Chem* 2000; 275: 823-7.
- [94] Troll W, Wiesner R, Frenkel K. 1987. Anticarcinogenic action of protease inhibitors. *Adv Cancer Res* 1987; 49: 265-83.
- [95] Birk Y. The Bowman-Birk Inhibitor. *Int J Pept Protein Res* 1985; 25: 113-31.
- [96] Yavelow J, Finlay T, Kennedy A, Troll W. Bowman-Birk soybean protease inhibitor as an anticarcinogen. *Cancer Res* 1983; 43S, 2454-9.
- [97] Stillmark PH. Ueber Ricin, ein giftiges Ferment aus den Samen von *Ricinus comm.* L. und einigen anderen Euphorbiaceen. Inaug. Diss., Dorpat University, 1888.
- [98] Armour JC, Perera RLC, Buchan WC, Grant G. Protease inhibitors and lectins in soya beans and effects of aqueous heat-treatment. *J Sci Food Agric* 1998; 78: 225-31.
- [99] Habermehl GG. Highlights on plant toxins in toxicon. *Toxicon* 2004; 44: 341-4.
- [100] Hartley MR, Lord JM. Cytotoxic ribosome-inactivating lectins from plants. *Biochim Biophys Acta* 2004; 1701: 1-14.
- [101] Lord JM, Roberts LM, Robertus JD. Ricin - structure, mode of action, and some current applications. *FASEB J* 1994; 8: 201-8.
- [102] Narayanan S, Surendranath K, Bora N, Suroli A, Karande AA. Ribosome inactivating proteins and apoptosis. *FEBS Lett* 2005; 579: 1324-31.
- [103] Carrasco L, Vazquez D, Hernandezlucas C, Carbonero P, Garciaolmedo F. Thionins - plant peptides that modify membrane-permeability in cultured mammalian-cells. *Eur J Biochem* 1981; 116: 185-9.
- [104] Giudici M, Poveda JA, Molina ML, de la Canal L, Gonzalez-Ros JM, Pfüller K, et al. Antifungal effects and mechanism of action of viscotoxin A(3). *FEBS J* 2006; 273: 72-83.
- [105] Bussing A, Suzart K, Bergmann J, Pfüller U, Schietzel M. Induction of apoptosis in human lymphocytes treated with *Viscum album* L. Is mediated by the mistletoe lectins. *Cancer Lett* 1996; 99: 59-72.
- [106] Smith DC, Salio M, Lord JM, Roberts LM, Cerundolo V. Lack of dendritic cell maturation by the plant toxin ricin. *Eur J Immunol* 2004; 34: 2149-57.
- [107] Rao PVL, Jayaraj R, Bhaskar ASB, Om K, Bhattacharya R, Parag S, et al. Mechanism of ricin-induced apoptosis in human cervical cancer cells. *Biochem Pharmacol* 2005; 69: 855-65.
- [108] Wélé A, Zhang YJ, Brouard JP, Poussot JL, Bodo B. Two cyclopeptides from the seeds of *Annona cherimola*. *Phytochemistry* 2005; 66: 2376-80.
- [109] Schmidt I, Renard D, Rondeau D, Richomme P, Popineau Y, Axelos MAV. Detailed physicochemical characterization of the 2S storage protein from rape (*Brassica napus* L.). *J Agric Food Chem* 2004; 52: 5995-6001.
- [110] Gehrig PM, Biermann K. Assignment of the disulfide bonds in napin, a seed storage protein from *Brassica napus*, using matrix-assisted laser desorption ionization mass spectrometry. *Pept Res* 1996; 9: 308-14.
- [111] Bérot S, Compoint JP, Larré C, Malabat C, Guéguen J. Large scale purification of rapeseed proteins (*Brassica napus* L.). *J Chromatogr B Biomed Sci Appl* 2005; 818: 35-42.
- [112] Ng TB, Ngai PHK. The trypsin-inhibitory, immunostimulatory and antiproliferative activities of a napin-like polypeptide from Chinese cabbage seeds. *J Pept Sci* 2004; 10: 103-8.
- [113] Ngai PHK, Ng TB. Phaseococcin, an antifungal protein with antiproliferative and anti-HIV-1 reverse transcriptase activities from small scarlet runner beans. *Biochem Cell Biol* 2005; 83: 212-20.
- [114] Ngai PHK, Ng TB. Coccin, an antifungal peptide with antiproliferative and HIV-1 reverse transcriptase inhibitory activities from large scarlet runner beans. *Peptides* 2004; 25: 2063-8.
- [115] Fitzgerald AJ, Jordinson M, Rhodes JM, Singh R, Calam J, Goodlad RA. Comparison of the effects of concanavalin-A and epidermal growth factor on epithelial cell proliferation in the rat intestine. *Aliment Pharmacol Ther* 2001; 15: 1077-84.
- [116] Ryder SD, Smith JA, Rhodes EGH, Parker N, Rhodes JM. Proliferative Responses Of HT29 And Caco2 Human Colorectal-Cancer Cells To A Panel Of Lectins. *Gastroenterology* 1994; 106: 85-93.
- [117] Follmer C, Barcellos GBS, Zingali RB, Machado OLT, Alves EW, Barja-Fidalgo C, et al. Canatoxin, a toxic protein from jack beans (*Canavalia ensiformis*), is a variant form of urease (EC 3.5.1.5): biological effects of urease independent of its ureolytic activity. *Biochem J* 2001; 360: 217-24.
- [118] Mejia EG, Valadez-Vega MDC, Reynoso-Camacho R, Loarca-Pina G. Tannins, trypsin inhibitors and lectin cytotoxicity in tepary (*Phaseolus acutifolius*) and common (*Phaseolus vulgaris*) beans. *Plant Foods Hum Nutr* 2005; 60: 137-45.
- [119] Wong JH, Ng TB. Vulgarinin, a broad-spectrum antifungal peptide from haricot beans (*Phaseolus vulgaris*). *Int J Biochem Cell Biol* 2005; 37: 1626-32.
- [120] Garcia-Gasca T, Salazar-Olivo LA, Mendiola-Olaya E, Blanco-Labra A. The effects of a protease inhibitor fraction from tepary bean (*Phaseolus acutifolius*) on *in vitro* cell proliferation and cell adhesion of transformed cells. *Toxicol In Vitro* 2002; 16: 229-33.
- [121] Campos JE, Whitaker JR, Yip TT, Hutchens TW, Blanco-Labra A. Unusual structural characteristics and complete amino acid sequence of a protease inhibitor from *Phaseolus acutifolius* seeds. *Plant Physiol Biochem* 2004; 42: 209-14.
- [122] Clemente A, Gee JM, Johnson IT, MacKenzie DA, Domoney C. Pea (*Pisum sativum* L.) protease inhibitors from the Bowman-Birk class influence the growth of human colorectal adenocarcinoma HT29 cells *in vitro*. *J Agric Food Chem* 2005; 53: 8979-86.
- [123] Lam SSL, Wang HX, Ng TB. Purification and characterization of novel ribosome inactivating proteins, alpha- and beta-pisavins, from seeds of the garden pea, *Pisum sativum*. *Biochem Biophys Res Commun* 1998; 253: 135-42.
- [124] Kennedy AR, Wan XS. Effects of the Bowman-Birk inhibitor on growth, invasion, and clonogenic survival of human prostate epithelial cells and prostate cancer cells. *Prostate* 2002; 50: 125-33.
- [125] Moy LY, Billings PC. A Proteolytic Activity In A Human Breast-Cancer Cell-Line Which Is Inhibited By The Anticarcinogenic Bowman-Birk Protease Inhibitor. *Cancer Lett* 1994; 85: 205-10.
- [126] Kennedy AR. The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. *Am J Clin Nutr* 1998; 68(Suppl): 1406S-12S.
- [127] Park JH, Jeong HJ, de Lumen BO. Contents and bioactivities of lunasin, Bowman-Birk inhibitor, and isoflavones in soybean seed. *J Agric Food Chem* 2005; 53: 7686-90.
- [128] de Lumen, BO. Lunasin: a cancer-preventive soy peptide. *Nutr Rev* 2005; 63: 16-21.
- [129] Park S, Ohba, H. Suppressive activity of protease inhibitors from buckwheat seeds against human T-acute lymphoblastic leukemia cell lines. *Applied Biochem Biotechnol* 2004; 117: 65-74.
- [130] Miyoshi N, Koyama Y, Katsuno Y, Hayakawa S, Mita T, Ohta T, et al. Apoptosis induction associated with cell cycle dysregulation by rice bran agglutinin. *J Biochem* 2001; 130: 799-805.
- [131] Dalla Pellegrina C, Matucci A, Zoccatelli G, Rizzi C, Vincenzi S, Veneri G, et al. Studies on the joint cytotoxicity of Wheat Germ Agglutinin and monensin. *Toxicol In Vitro* 2004; 18: 821-7.

- [132] Pusztai A, Ewen Swb, Grant G, Brown Ds, Stewart Jc, Peumans WJ, *et al.* Antinutritive effects of wheat-germ-agglutinin and other N-acetylglucosamine-specific lectins. *Brit J Nutr* 1993; 70: 313-21.
- [133] Vincentini O, De Angelis I, Iannuccelli R, Silano M, Stammati A, De Vincenzi M. Protective effects of mannan in Caco-2/TC7 cells treated with wheat-derived peptides. *Carbohydr Polym* 2005; 62: 338-43.
- [134] Giovannini C, Mancini E, De Vincenzi M. Inhibition of the cellular metabolism of Caco-2 cells by prolamin peptides from cereals toxic for coeliacs. *Toxicol In Vitro* 1996; 10: 533-8.
- [135] Mendez E, Rocher A, Calero M, Girbes T, Citores L, Soriano F. Primary structure of omega-hordothionin, a member of a novel family of thionins from barley endosperm, and its inhibition of protein synthesis in eukaryotic and prokaryotic cell-free systems. *Eur J Biochem* 1996; 239: 67-73.
- [136] Sampson HA. Update on food allergy. *J Allergy Clin Immunol* 2004; 113: 805-19.
- [137] Bock SA, Munoz-Furlong A, Sampson HA. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol* 2001; 107: 191-3.
- [138] Moneret-Vautrin DA, Morisset M, Flabbee J, Beaudouin E, Kanny G. Epidemiology of life-threatening and lethal anaphylaxis: a review. *Allergy* 2005; 60: 443-51.
- [139] EFSA, Opinion of the Scientific Panel on dietetic products, nutrition and allergies on a request from the commission relating to the evaluation of allergenic foods for labelling purposes. The EFSA Journal 32, 1-197. (http://www.efsa.eu.int/science/nda/nda_opinions/catindex_en.html; access date 2006-02-27).
- [140] Eggesbo M, Botten G, Halvorsen R, Magnus P. The prevalence of CMA/CMPI in young children: the validity of parentally perceived reactions in a population based study. *Allergy* 2001; 56: 393-402.
- [141] Laoprasert N, Wallen ND, Jones RT, Hefle SL, Taylor SL, Yunginger JW. Anaphylaxis in a milk-allergic child following ingestion of lemon sorbet containing trace quantities of milk. *J Food Prot* 1998; 61: 1522-4.
- [142] Fremont S, Kanny G, Bieber S, Nicolas JP, Moneret-Vautrin DA. Identification of a masked allergen, alpha-lactalbumin, in baby-food cereal flour guaranteed free of cow's milk protein. *Allergy* 1996; 51: 749-54.
- [143] Nowak-Węgrzyn A, Shapiro GG, Beyer K, Bardina L, Sampson HA. Contamination of dry powder inhalers for asthma with milk proteins containing lactose. *J Allergy Clin Immunol* 2004; 113: 558-60.
- [144] Bindselev-Jensen C, Briggs M, Osterballe M. Can we determine a threshold level for allergenic foods by statistical analysis of published data in the literature? *Allergy* 2002; 57: 741-6.
- [145] Brodard V, Bernard H, Wal JM, David B, Peltre G. Two-dimensional analysis of cow's milk allergens with the ITPG-DALT technique. In: McGhee J, Mestecky J, Tlaskalova H, Sterzl J Eds, Recent advances in mucosal immunology, series advances in experimental medicine and biology, Plenum Press, New York 1995; 875-8.
- [146] Wal JM. Cow's milk proteins/Allergens. *Ann Allergy Asthma Immunol* 2002; 89(Suppl): 3-10.
- [147] Selo I, Clement G, Bernard H, Chatel JM, Creminon C, Peltre G, *et al.* Allergy to bovine β -lactoglobulin: specificity of human IgE to tryptic peptides. *Clin Exp Allergy* 1999; 29: 1055-63.
- [148] Fritsche R, Adel-Patient K, Bernard H, Martin-Paschoud C, Schwarz C, Ah-Leung S, *et al.* IgE mediated rat mast cell triggering with tryptic and synthetic peptides of bovine β lactoglobulin. *Int Arch Allergy Immunol* 2005; 138: 291-7.
- [149] Haddad ZH, Kalra V, Verma S. IgE antibodies to peptic and peptic-tryptic digests of β -lactoglobulin: significance in food hypersensitivity. *Ann Allergy* 1979; 42: 368-71.
- [150] Maynard F, Jost R, Wal JM. Human IgE binding capacity of tryptic peptides from bovine alpha-lactalbumin. *Int Arch Allergy Immunol* 1997; 113: 478-88.
- [151] Jarvinen KM, Chatchatee P, Bardina L, Beyer K, Sampson HA. IgE and IgG binding epitopes on α -lactalbumin and β -lactoglobulin in cow's milk allergy. *Int Arch Allergy Immunol* 2001; 126: 111-8.
- [152] Adel-Patient K, Nahori MA, Proust B, Silva JRLE, Creminon C, Wal JM, *et al.* Elicitation of the allergic reaction in β lactoglobulin sensitized Balb/c mice : biochemical and clinical manifestations differ depending on the structure of the BLG used for the challenge. *Clin Exp Allergy* 2003; 33: 376-85.
- [153] Bernard H, Negróni L, Chatel JM, Clement G, Adel-Patient K, Peltre G, *et al.* Molecular basis of IgE cross-reactivity between human β -casein and bovine β -casein, a major allergen of milk. *Mol Immunol* 2000; 37: 161-7.
- [154] Meisel H, Bernard H, Fairweather-Tait S, FitzGerald RJ, Hartmann R, Lane CN, *et al.* Detection of caseinophosphopeptides in the distal ileostomy fluid of human subjects. *Brit J Nutr* 2003; 89: 351-8.
- [155] Spuergerin P, Mueller H, Walter M, Schiltz E, Forster J. Allergenic epitopes of bovine alpha_{s1}-casein recognized by human IgE and IgG. *Allergy* 1996; 51: 306-12.
- [156] Chatchatee P, Jarvinen K, Bardina L, Beyer K, Sampson H. Identification of IgE- and IgG- binding epitopes on α _{s1}-casein: Differences in patients with persistent and transient cow's milk allergy. *J Allergy Clin Immunol* 2001; 107: 379-83.
- [157] Sakaguchi H, Inoue R, Kaneko H, Watanabe M, Suzuki K, Kato Z, *et al.* Interaction among human leucocyte antigen-peptide-T cell receptor complexes in cow's milk allergy: the significance of human leucocyte antigen and T cell receptor-complementarity determining region 3 loops. *Clin Exp Allergy* 2002; 32: 762-70.
- [158] Wal JM. Thermal processing and allergenicity of foods. *Allergy* 2003; 58: 727-9.
- [159] Host A, Samuelsson EG. Allergic reactions to raw, pasteurized, and homogenized/pasteurized cow milk: a comparison. A double-blind placebo-controlled study in milk allergic children. *Allergy* 1988; 43: 113-8.
- [160] Astwood JD, Leach JN, Fuchs RL. Stability of food allergens to digestion *in vitro*. *Nat Biotechnol* 1996; 14: 1269-73.
- [161] Fu TJ, Abbott UR, Hatzos C. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid-a comparative study. *J Agric Food Chem* 2002; 50: 7154-60.
- [162] Diaz-Perales A, Blanco C, Sanchez-Monge R, Varela J, Carrillo T, Salcedo G. Analysis of avocado allergen (Prs a 1) IgE-binding peptides generated by simulated gastric fluid digestion. *J Allergy Clin Immunol* 2003; 112: 1002-7.
- [163] Hong SJ, Michael JG, Fehrer A, Leung DY. Pepsin-digested peanut contains T-cell epitopes but no IgE epitopes. *J Allergy Clin Immunol* 1999; 104: 473-8.
- [164] Oldaeus G, Björkstén B, Emarsson R, Kjellman NIM. Antigenicity and allergenicity of cow milk hydrolysates intended for infant feeding. *Pediatr Allergy Immunol* 1991; 2: 156-64.
- [165] Ragno V, Giampietro PG, Bruno G, Businco L. Allergenicity of milk protein hydrolysate formulae in children with cow's milk allergy. *Eur J Pediatr* 1993; 152: 760-2.
- [166] De Boissieu D, Matarazzo P, Dupont C. Allergy to extensively hydrolyzed cow's milk proteins in infants: identification and treatment with an amino acid-based formula. *J Pediatr* 1997; 131: 744-7.
- [167] Eiwegger T, Rigby N, Mondoulet L, Krauth MT, Boehm A, Dehlink E, *et al.* Gastro-duodenal digestion products of the major peanut allergen Ara h1 retain an allergenic potential. *Clin Exp Allergy* 2006; 36: 1281-8.
- [168] Codex Alimentarius. Foods derived from biotechnology. Guidelines for the conduct of food safety assessment of foods derived from recombinant-DNA plants. CAC/GL 45-2003.
- [169] EFSA. Guidance document of the Scientific Panel on genetically modified organisms for the risk assessment of genetically modified plants and derived food and feed. EFSA Journal 2004; 99: 1-94. (http://www.efsa.eu.int/science/gmo/gmo_guidance/660_en.html; access date 2006-02-27).
- [170] WHO/FAO, 2001. FAO/WHO expert consultation on foods derived from biotechnology. Evaluation of allergenicity of genetically modified foods. WHO/FAO, January 2001, Rome. (http://www.who.int/foodsafety/publications/biotech/en/ec_jan2001.pdf; access date 2006-02-27).
- [171] Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. OJ, L308, 25.11.2003, 15-8.
- [172] Leduc V, Moneret-Vautrin DA, Guerin L, Morisset M, Kanny G. Anaphylaxis to wheat isolates: Immunochemical study of a case proved by means of double-blind, placebo-controlled food challenge. *J Allergy Clin Immunol* 2003; 111: 897-9.
- [173] Denery-Papini S, Nicolas Y, Popineau Y. Efficiency and limitations of immunochemical assays for the testing of gluten-free foods. *J Cereal Sci* 1999; 30: 121-31.

- [174] Pecquet C and Lauriere M. Hydrolysats de proteines du blé: nouveau allergens [Wheat protein hydrolysates: New allergens]. *Rev Fr Allergol Immunol Clin* 2003; 43: 21-3.
- [175] Opinion of the NDA Panel related to a notification on hydrolysed wheat gluten used as fining agent in wines pursuant to Article 6 Paragraph 11 of Directive 2000/13/EC. *EFSA Journal* 2004; 148: 1-8.
- [176] Opinion of the NDA Panel related to a notification on acid hydrolysed vegetable protein from soy pursuant to Article 6 paragraph 11 of Directive 2000/13/EC. *EFSA Journal* 2005; 190: 1-6.
- [177] McIntosh GH, Le Leu RK. The influence of dietary proteins on colon cancer risk. *Nutr Res* 2001; 21: 1053-66.
- [178] Gustafsson L, Hallgren O, Mossberg AK, Pettersson J, Fischer W, Aronsson A, *et al.* HAMLET kills tumor cells by apoptosis: Structure, cellular mechanisms, and therapy. *J Nutr* 2005; 135: 1299-303.
- [179] Ekwall B. Overview of the final MEIC results: II. The *in vitro* - *in vivo* evaluation, including the selection of a practical battery of cell tests for prediction of acute lethal blood concentrations in humans. *Toxicol In Vitro* 1999; 13: 665-73.
- [180] Salamat-Miller N, Johnston TP. Current strategies used to enhance the paracellular transport of therapeutic polypeptides across the intestinal epithelium *Int J Pharm* 2005; 294: 201-16.
- [181] International Organization for Standardization. DIN EN ISO 10993-5: Biological evaluation of medical devices -- Part 5: Tests for *in vitro* cytotoxicity 1999.
- [182] Narai A, Arai S, Shimizu M. Rapid decrease in transepithelial electrical resistance of human intestinal caco-2 cell monolayers by cytotoxic membrane perturbants. *Toxicol In Vitro* 1997; 11: 347-54.
- [183] Rossi A, Poverini R, DiLullo G, Modesti A, Modica A, Scarino ML. Heavy metal toxicity following apical and basolateral exposure in the human intestinal cell line Caco-2. *Toxicol In Vitro* 1996; 10: 27-31.
- [184] DuBuske LM. IgE, allergic diseases, and omalizumab. *Curr Pharm Des* 2006; 12(30): 3929-44.
- [185] Saleh F, Renno W, Klepacek J, Ibrahim G, Dashti H, Asfar S, *et al.* Direct evidence on the immune-mediated spontaneous regression of human cancer: an incentive for pharmaceutical companies to develop a novel anti-cancer vaccine. *Curr Pharm Des* 2005; 11(27): 3531-43.
- [186] Schaedel O, Reiter Y. Antibodies and their fragments as anti-cancer agents. *Curr Pharm Des* 2006; 12(3): 363-78.