INCO COPERNICUS News

Issue 1

Quality Control in Dairy Industry

January 1999

Editorial

Thank you for reading this newsletter concerning the activities of the participants of the EU-funded Copernicus programme, "Rapid, specific detection of *Listeria monocytogenes* by antibody-based techniques and on-line sensor technology; development of improved control of food safety for industry and the consumer". The project brings together academic and industrial partners from Czech Republic, Ireland, Slovakia and the United Kingdom.

The food industry is currently responding to the demands presented by changing consumer tastes, the introduction of novel production and the appearance of new foodborne pathogens. Rapid, sensitive and specific microbiological analysis is of fundamental importance in the response to such challenges. Until recently the basic principles of microbial identification had hardly changed since the time of Pasteur, although modern technology has streamlined the process. It is widely accepted that these approaches, based on the recognition of phenotypic differences, do not satisfy the criteria of speed and sensitivity. They are normally employed retrospectively and offer little chance to influence the application of control procedures during a production run. Techniques capable of a more rapid response would be of major benefit in terms of providing safer products, responding more quickly to problems associated with changing production methods, and ultimately reducing costs of processed foods.

The use of antibodies in vitro in order to analyse foodstuffs is now well accepted, and can lead to one of the new fast techniques for detection of microbial contamination. This project will allow the method developed to be disseminated widely through both academia and industry, and thus should improve the likehood of this method becoming more widely used as an alternative to traditional methods. Implementation of Hazard Analysis Critical Control Point (HACCP) concept in East European countries needs such rapid and specific methods. The net result of using these assays should improved the quality of foods. Participants will transfer skills and knowledge between each other not only through this newsletter, but through regular project meetings and workshops (intra- and international in nature). If you require any further information on any of these events, do not hesitate to contact any of the participants.

Pavel Rauch, coordinator

Aims, objectives and expected results of INCO COPERNICUS project PL NO 979012

"Rapid, specific detection of Listeria monocytogenes by antibody-based techniques and on-line sensor technology; development of improved control of food safety for industry and the consumer".

The overall objective of this EU founded project is to develop rapid, specific test methods for the detection of *Listeria in foods*, with the aim of introducing appropriate quality control into food production processes of the CCE partners. This will lead to improved food safety, greater consumer confidence in the industry and will meet the requirements of EU legislation, thus allowing export to EU countries. The methodology, of course, will also be appropriate to quality control system within EU countries, and could well be introduced to producers there, with considerable benefit.

The methods will be based on antibodies which are very specific to *L. monocytogenes* (and, as a minor objective, to *L. ivanovii*). These antibodies will be incorporated into two very different types of detection system. The first type will be solid-phase immunoassays (probably more familiar to many readers as ELISAs) in several different formats, including microtitration plates and dipsticks, intended for analysis of samples in the laboratory; the second type will be very different, a biosensor for on-line use in production processes. Each of these methods could be readily incorporated into a HACCP or other quality control system. Both types of method will be designed to be easy to use and rapid, with the intention of producing a result in much less than 48 hours.

Of course, previous attempts have been made to develop rapid methods for *Listeria*, but these have all suffered disadvantages; in particular, those based on nucleic acid detection (such as probes or PCR methods) are poorly suited for use in routine diagnostic situations. Antibody-based tests are very flexible and much more user-friendly.

A commercially available immunoassay has shown promissing sensitivity, but this assay also detects *Listeria* innocua, a non-pathogenic species. The use of biosensors for the pathogen has not been published. Antibody-based techniques are the most convenient rapid methods in current use, and are the methods of our choice here.

The quality of the antibodies used will be crucial to the success of the project. In particular, it is necessary that they are absolutely specific to the target organism, and do not bind to other, irrelevant, bacteria. Therefore, we have decided that the antibodies we use will be designed to recognise the pathogenicity proteins. Several of these proteins are unique to *L. monocytogenes*; indeed, they are what makes the organism what it is! Taking this a stage further, because the amino acid sequences of these proteins are already known, we are able to select regions from those sequences that are central to the pathogenic process, and are particularly good for antibody production. These regions of the protein form peptides of 10—15 amino acids, and we will synthesise these peptides (see next page) and use them for antibody production. We can, then, be sure that we are targeting something unique.

An important element of the project is the use of the newly-developed assays in a real industrial situation. After laboratory-scale trials, process workers at the industrial partners will be trained in the use of the methods and will compare them to the normal quality control procedures. In this manner, the assays will be validated before being released for wider use. Additionally, the results of the project will be passed on to other potential users via scientific meetings and publicity material.

Our meeting in May in Prague. From left to right: R. O'Kennedy, L. Karasová, L. Fukal, G. Brett, G. Wyatt, M. Morgan, P. Rauch, A. Šovčíková, K. Horáková, V. Boroš, I. Hochel, P. Roubal. Pictured by M. Tomáška.

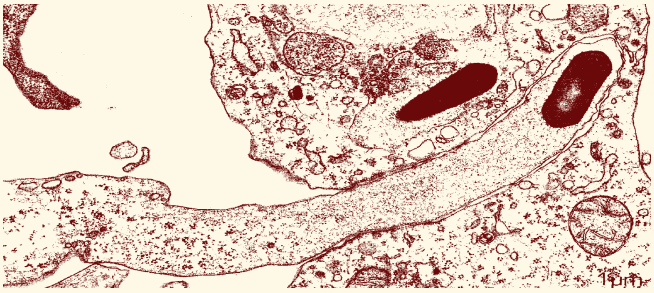
The seven partners in the project are based in four countries. The co-ordinating laboratory is at the Institute of Chemical Technology in Prague (Czech Republic) where the Dairy Research Institute is also located. An accredited Reference Laboratory in the State Veterinary Institute in Jihlava is another Czech participant. In Slovakia, the Slovak Technical University in Bratislava and the Dairy Research Institute in Zilina are partners. Dublin City University (Ireland), and the Institute of Food Research, Norwich (United Kingdom) are the Western participants. The members of this Consortium have collaborated previously on various EU-funded projects, and this previous contact and experience will certainly allow the project to make rapid progress and, we hope, reach a successful conclusion.

Overview of Listeria

Not until the 1980s was it recognised that *Listeria monocytogenes* could be transmitted to humans in food. Previously, the source of infection either remained unknown, or was found to be from an animal reservoir (i.e. a zoonosis, especially in farm and slaughter-house workers), or via cross-infection acquired in hospital especially in compromised patients.

Listeria are rod-shaped bacteria, which are motile and do not produce spores. They have no special heat-resistance; however, the ability to grow at refrigeration temperatures (4 °C) is an important property of these organisms. Currently, there are six accepted species: *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri*; of these, *L. monocytogenes* is the principal human pathogen, with only a few known cases of human listeriosis due to other species, mainly *L. ivanovii* (an animal pathogen). The true habitat of *Listeria* is probably decaying plant material (as indicated by its ability to grow at low temperatures and motility only below 30 °C), but it has world-wide distribution in the environment, especially in water, soil and vegetable matter. A wide range of symptom-less animal carriers are known, including mammals, fish, birds and invertebrates. Human carriage rates have been estimated at 5—10% (in faeces and respiratory tract) and therefore the organism is easily found in sites liable to contamination with human or animal sewage.

a)



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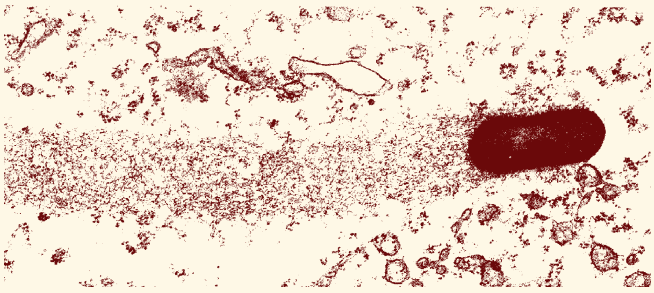


Ultrathin sections through L. monocytogenes infected macrophage showing a) protuberance containing bacterium, b) typical actin tail.
Pictures from: Cossart, P. (1995) Actin-based motility. Current Opinion in Cell Biology 7, 94—101.

As mentioned above, it was only relatively recently that food was shown to be a vehicle of transmission for *Listeria monocytogenes*. This was finally recognised when epidemiological studies of several outbreaks in the 1980s pointed to certain foods as a common factor. Since then, it has been shown that dairy foods (especially soft cheeses) have a stronger association with listeriosis than any other food group (enteric contamination); outbreaks have also been linked with salads, vegetables and coleslaw (irrigation with polluted water), and meats, especially poultry (again enteric contamination). A particular concern has been long shelf-life cook-chill ready meals, because of the low growth temperature of the organism.

There are various regulatory requirements covering *Listeria* in food, with many countries having a zero tolerance (<1 cell in 50g food) for *L. monocytogenes* in certain foods. Within the European Union (EU), the Commission makes decisions on principles of food law, but it is the duty of member states to provide legislation to implement those principles. In the United Kingdom, for example, the Food Safety Act, 1990, makes it an offence "to supply food that is injurious to health, unfit for human consumption or contaminated". Under that Act, "analysis" is defined as any technique establishing the composition of food, and thus, in theory, the suppliers of a food can choose whatever method of analysis they wish, provided that they meet the requirements of the Act. It seems that both the EU and the United States Food and Drug Administration have accepted that the best approach to food safety management is the Hazard Analysis Critical Control Point (HACCP) system, and are in the process of moving that system from voluntary to mandatory status.

Listeria as pathogens

In humans, there are two distinct phases of *Listeria* infection. The first, the enteric phase, is a mild, influenza-like illness, and may be accompanied by slight fever; the incubation period is thought to vary from 4 days to several weeks, and this phase may go un-diagnosed. Susceptible individuals may go on to develop the second, listeriosis, phase which is much more serious and has a considerable mortality rate; in this phase there is systemic inflammation and necrosis of tissue, and meningitis and septicaemia can follow. In pregnant women, foetal infection and abortion are common complications of *Listeria* infection. Whilst *Listeria* infections are less common that most other foodborne diseases, the severe nature of the infection, especially in immunocompromised individuals, makes it a major cause of concern for food suppliers.

L. monocytogenes has a fascinating and complex mechanism of pathogenicity. It infects tissues by a direct cell-to-cell route, and is able to enter and multiply in several types of cells. The primary site of entry is still not known for certain, but it is probably via the epithelial cells that form the lining of the small intestine. It is then known to become internalised into macrophages (large cells which are part of the defence system of the body), and then to pass via the lymphatic circulation to the liver and spleen during the listeriosis phase; liver cells are a major site of multiplication of the organism.

L. monocytogenes is able to achieve all this because it produces several kinds of 'pathogenicity proteins' on the surface of the organism. The first of these, internalin, enables the bacteria to enter epithelial cells, mentioned above as the point of access to the body. Once inside a cell, the bacterium is still enclosed in a vesicle, but the membranes of this are ruptured by two other proteins, listeriolysin and phospholipase C.

The bacteria are now in the cytoplasm of the host cell, and move through this cell pushed along by a 'tail' of filaments which is constructed with the help of another of these proteins, the actin-assembly protein. On reaching the cell wall, this is then protruded into the next cell, the protrusion is ruptured and the process repeated. This is a simplified account of the infection, and several other pathogenicity proteins have been identified; it is almost certain that other aspects of the pathogenesis mechanism remain to be discovered.

Gary M. Wyatt

The first planning project meeting

The planning meeting of the INCO COPERNICUS Project PL 979012 "Rapid, specific detection of *Listeria monocytogenes* by antibody-based technoques and on-line sensor technology" was held in Prague in June 1998 before the official starting of the project to settle down organisation details of the work and discuss possible problems occuring in the first period of the project and we used the opportunity to meet personally all participants. Following meeting minutes describe the part of subjects discussed during these two days in Prague (June 23rd and 24th 1998).

Present: *Pavel Rauch (Prague; project coordinator), Mike Morgan, Gary Wyatt, Gary Brett (IFR), Richard O'Kennedy (Dublin), Katarína Horáková, Vladimír Mastihuba, Andrea Šovčík ová (Bratislava), Vladimír Boroš, Martin Tomáška (Žilina), Igor Hochel, Ladislav Fukal, Ludmila Karasová (Prague), Petr Roubal (Milcom), Josef Brychta (Jihlava).*

Introduction

Welcome given by Pavel Rauch (PR).

Mike Morgan (MM) spoke on previous collaboration. He stressed that Brussels likes partners who have worked together before, and mentioned the EU Fifth Framework Programme. He said that papers and posters were very important, and that the EU must be acknowledged in these. He suggested that extra money might be available from the EU for mobility of staff/students connected with the project.

Discussion followed on the current protocols in use in the Czech and Slovak republics.

Gary Wyatt (GW) gave an overview of Listeria, including its occurrence in foods, pathogenicity mechanism including the proteins that are intended to be the target for antibodies in this project, current detection methods, legislation in the EU, the requirements of an alternative detection method, and HACCP.

Roles of the various partners

Institute of Chemical Technology, Prague (P1), CZ

Pavel Rauch informed about the role of his group. In the frame of the project he will produce rabbit antisera using the peptides and whole *Listeria* proteins as immunogens and characterise the antibodies.

Institute of Food Research, Norwich (P2), UK

Gary Brett (GB) described the use of synthetic peptides, including MAPs, as immunogens and gave examples from IFR's work. He went on to outline the procedures for peptide synthesis, including restrictions imposed by certain sequences. GW showed some possible sequences from the target proteins (internalins, actin-assembly protein, p60 protein, and others), and discussed the basis for selection of those sequences. He suggested that, to back this up, whole proteins should also be used as immunogens.

Dublin City University (P4), IRL

Richard O'Kennedy (RO'K) described the use of various sensor surfaces, and of the BiaCore instrument. He said that the machine was particularly good for characterising antibodies, especially selecting on the basis of stability and affinity with a view to constructing either disposable or re-usable biosensors. He said that polyclonal antisera can be very good in these systems, and that monoclonals are not an absolute requirement. He described his smaller, cheaper sensors, in particular a probe system, which have been derived from BiaCore and Texas products. He discussed the problem of non-specific binding in food matrices, sample preparation and biosensor regeneration.

Slovak Technical University, Bratislava (P3), SK

Katarína Horáková (KH) mentioned the need to equip her Laboratory to handle *Listeria*. She will optimise cultural conditions for expression of the chosen protein, but needs an ELISA as soon as possible to assess this. She asked who would prepare the whole proteins to be used as immunogens. GW said that Norwich could probably do this. There was discussion on a stable preparation of *Listeria* cells to be used in standardising assays developed by different partners e.g. a freeze-dried preparation. GW would investigate this idea. Discussion followed on what quantities of materials would be required — GB said that production of 100 mg of MAPs were no problem, and it seems that mg amounts of the whole proteins will be needed.

It was suggested that the main food products to be tested would be raw milk and soft cheese, but that this decision was best left until later in the project.

Milcom — Dairy Research Institute, Prague (P5), CZ

Peter Roubal (Pro) talked about the classification of raw milk in the Czech Republic, based on levels of various bacteria present, and said that, from 1/1/98, standards were the same as in the EU. He did not have any data on *Listeria* content, but said that Josef Brychta (JB, who had had to leave the meeting) has figures for raw milk, cheeses and ham. He had one main request for the project: to produce a simple, sensitive and cheap assay for use in HACCP.

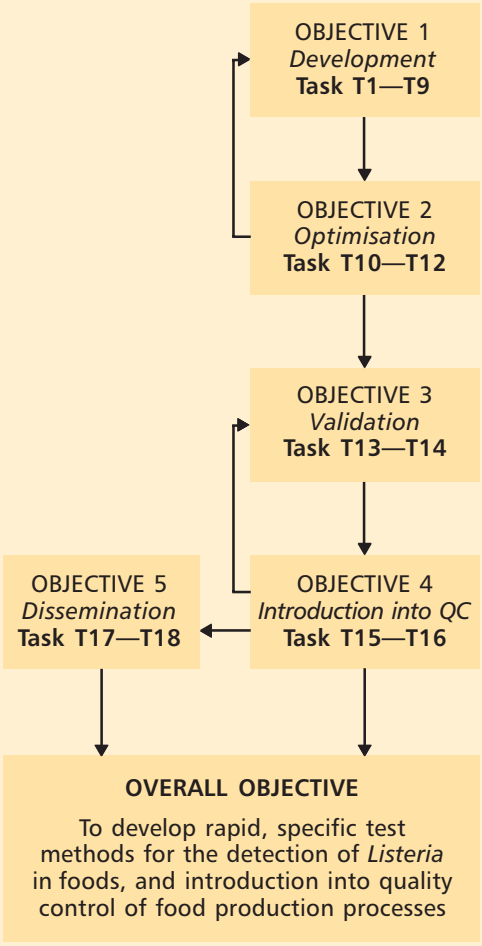
Dairy Research Institute, Žilina (P6), SK

Martin Tomáška (MT) said that in the Slovak Republic, obligatory examination of various products for *Listeria monocytogenes* is a new requirement. There is a Codex Alimentarius and an ISO standard procedure, taking more than 5 days. He suggested that the selective propagation medium in that procedure could be used as a starting point for KH's work.

Future Meetings will be

April 1999	Žilina
September 1999	Norwich (with Agri Food Antibodies '99 meeting)
May 2000	Dublin

Tasks of programme



Institute of Chemical Technology, Prague **P1**

MILCOM-Dairy Research Institute, Prague **P5**

BBSRC Institute of Food Research, Norwich **P2**

Slovak Technical University, Bratislava **P3**

Dairy Research Institute, Žilina **P6**

School of Biological Sciences, Dublin **P4**

Task	Time period	Partner	Description
T1	months 0—3	**P1/P2**	Identify suitable cell-surface proteins and suitable peptide sequences within those proteins
T2	months 2—6	**P2**	Construct synthetic peptides, based on above, for use as immunogens (coupled to carrier proteins if necessary)
T3	months 4—12	**P1**	Immunise rabbits on suitable time schedule; collect antisera
T4	months 8—14	**P1/P4**	Characterise and select suitable antisera; purify if necessary
T5	months 13—14	**P4**	Immobilise chosen antibodies to Biacore sensor chip
T6	months 14—18	**P4**	Apply the biosensor to detection of *L. monocytogenes* in model systems
T7	months 15—24	**P4**	Repeat **T5** & **T6** using the new single-use sensor chips and more user-friendly system currently under development
T8	months 13—24	**P1/P2**	Use chosen antibodies to develop ELISAs for the pathogen; investigate a range of potential formats for these assays e.g. microtitre plates, dipsticks, magnetic beads
T9	months 19—24	**P1**	Apply the ELISAs to detection of *L. monocytogenes* in model systems
T10	months 7—12	**P3**	Consider factors controlling expression of the chosen target protein in *L. monocytogenes;* develop culture media in which these factors are optimised, allowing for the presence of food materials in samples
T11	months 13—18	**P3**	Use the new optimised culture media for growth of *L. monocytogenes* in pure and mixed cultures: receive training in the assays developed by other partners (**P1 & P4**) and apply these assays to the cultures
T12	months 19—24	**P3**	Repeat **T11** using both inoculated and artificially-contaminated food samples
T13	months 25—27	**P3/P5/P6**	Analysts to train in the new system at the laboratory of partner **P3**
T14	months 26—30	**P5/P6**	Analysts to apply the new systems in their own laboratories, and make validation studies to compare performance with classical techniques
T15	months 28—29	**P5/P6**	Investigate the point of application of the new tests in industrial situations, as part of GMP/HACCP systems
T16	months 29—36	**P5/P6**	Training of process workers and application of the tests in the identified quality control system; validation of the tests compared to current methods in the factory
T17	months 13—36	**all**	Dissemination of results
T18	months 34—36	**P1**	Application for funding from Accompanying Measures to hold workshop for reporting/discussion of the complete project with other potential industrial users

Production of antibodies to proteins — the use of synthetic peptides

Proteins are common targets for the generation of antibodies and a number of approaches are possible, exploiting the primary, secondary and tertiary structure. The immunogen should be at least 1kDa, approximately 10 amino acids. Immunisation with purified protein is often the most reliable route, although any homology with related proteins may lead to unwanted cross-reactions.

Protein fragments can be produced by proteolytic degradation or by chemical synthesis. Conjugating shorter peptides to carrier proteins is also a commonly used approach. However, the conjugation reaction is difficult to quantify and antibodies are also often generated to the carrier.

An alternative method is to immunise with multi-antigenic peptides (MAPs), synthetic molecules carrying up to eight copies of the chosen peptide. MAPs are constructed around a lysine core which itself is too small to be significantly immunogenic. The whole molecule may have a molecular weight of many thousand, composed of multiple copies of the target sequence.

Peptide Synthesis

The technique of peptide synthesis changed dramatically in the early 1960s with the introduction of solid phase peptide synthesis (SPPS) a procedure in which the growing peptide chain is elongated while attached through the carboxy terminus to a stable, insoluble solid support, allowing removal of unreacted reagents by simple washing and filtration.

Peptide bond formation does not occur spontaneously under normal conditions so one of the groups, usually the carboxyl, is converted to a reactive form, for example an activated pentofluorophenyl ester. A huge array of protecting groups has been developed to block the functional groups of individual amino acids during synthesis, preventing their participation in unwanted side reactions. The amino group of the residue being added is also blocked to prevent uncontrolled polymerisation. Common amino-protecting groups are tertiary butyloxycarbonyl (Boc) and fluoromethoxycarbonyl (Fmoc), cleaved by strong acid and mild organic base respectively. An outline of the continuous flow, automated synthesis protocol used at IFR is given in Fig.

The C-terminal amino acid is attached to the solid support via a labile linker group. The N-a-protecting group, Fmoc is removed in weak base, 20% piperidine in dimethylformamide, its removal monitored spectrophotometrically at 300 nm. After a thorough washing step to remove residual piperidine the next amino acid is added as an activated ester with the a-amino group and if necessary, side groups suitably protected. Not all functional groups need protecting and furthermore the reactivity of some are dependant on peptide sequence. Complete coupling is ensured by counterion distribution monitoring (CDM). This system is based on the weak interaction between dye molecules and —NH_2 groups. The relative amounts of bound and free dye, monitored spectrophotometrically, indicate the completeness of reaction. After coupling and a thorough washing step, Fmoc is removed and the cycle repeated until the entire peptide has been synthesised.

Finished peptide is cleaved from the support using suitable reagents, generally trifluoroacetic acid mixtures which also cleaves the side chain protecting groups. To prevent reattachment, protecting groups are removed from solution by the inclusion of appropriate scavengers such as water, ethanedithiol or diisopropylsilane. The peptide is isolated by precipitation in diethyl ether and filtration.

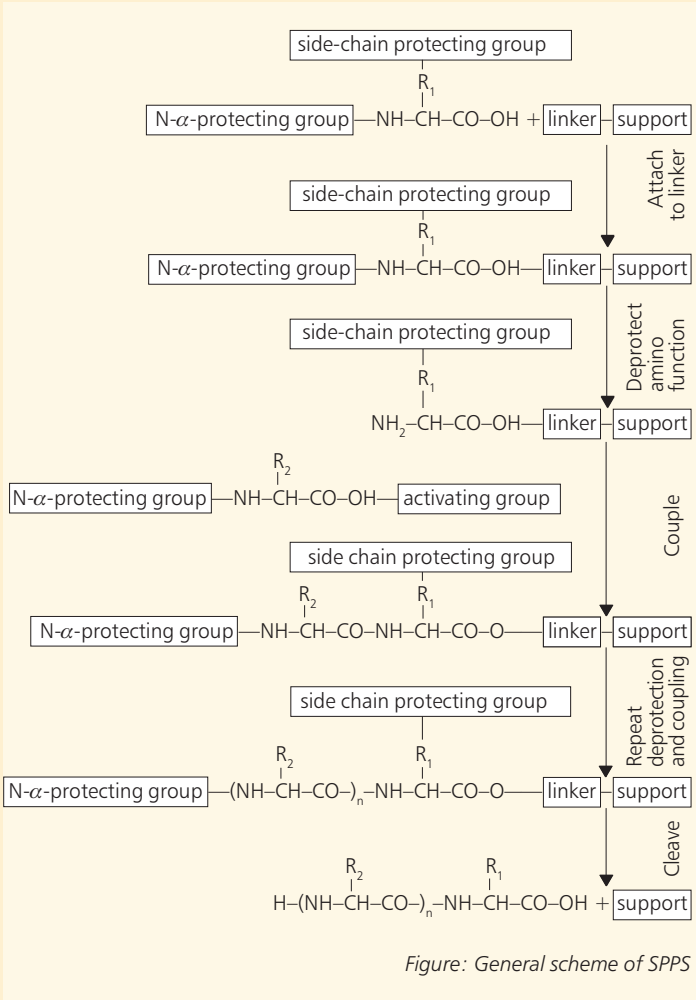


Figure: General scheme of SPPS

Purity and integrity can be checked by reverse phase HPLC and mass spectrometry.

In general terms the procedure for synthesis of MAPs is similar, the main difference being in the coupling of the first residue to the support. The lysine core can either be purchased attached directly to a resin or built synthetically with careful choice of protecting groups on both the α- and ε-amino groups of lysine. Peptides are then synthesised directly onto this core. An alternative approach is to make the peptide in the usual way and couple it to the lysine core after completion, allowing a greater degree of control and monitoring during and after synthesis. Similarly it may be easier to purify and determine peptide quality at this stage than after synthesis of the MAP.

By careful choice of sequence, immunogen and synthesis protocol, synthetic peptides can be used not only in the production of specific andibodies but also in screening assays and antibody characterisation. With the advent of automated synthesisers and the ever expanding array of activated amino acids, protecting groups and synthesis protocols, the opportunities for use of peptides are virtually endless. As well as branched MAPs it is possible to prepare linear or derivatised MAPs. Constrained cyclic peptides can be made to mimic the conformation displayed by the peptide within the protein. With rigorous control of each synthesis step there is often no need for extensive purification but when required peptides can be highly purified and are stable, reproducible, chemically defined molecules.

Gary Brett

Notice board

NEW INTERNATIONAL SCIENTIFIC PROJECTS

We are looking for partners wishing to cooperate within following scientific project:

The role of tetrodotoxin-sensitive Na^+ channel in non-excitable cells

Address: *Dr. Ľudovít Varečka, Assoc. Prof., Slovak Technical University, Faculty of Chemical Technology, Department of Biochemistry and Microbiology, Radlinského 9, 812 37 Bratislava, Slovak Republic,*
fax: +421–7–39 31 98
e-mail: varel@chtf.stuba.sk; varecka@checdek.chtf.stuba.sk

Lactobacilli in food technology

Topics of project: *lactic acid fermented vegetables, probiotic properties of Lactobacilli, reduction of nitrates and nitrites by Lactobacilli, analysis of organic acids in lactic acid fermented foods*

Address: *Dr. Eva Hybenová, Slovak Technical University, Faculty of Chemical Technology, Department of Food Chemistry and Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic,*
tel: +421–7–593 25 550, fax: +421–7–39 31 98

Natural antioxidants for food

Aims of project: *Isolation and investigation of plant antioxidants; optimisation of antioxidant composition of food preservative blends*

Address: *Dr. Stanislav Sekretár, Slovak Technical University, Faculty of Chemical Technology, Department of Milk, Fats and Food Hygiene, Radlinského 9, 812 37 Bratislava, Slovak Republic*
fax: +421–7–39 31 98, e-mail: sekretar@chtf.stuba.sk

Use of transglycosylation reactions in the synthesis of biologically important oligosaccharides

Research group at the **Department of Biochemistry of the University of Trieste** (Italy) is equally open to collaboration/cooperation both with academia and industry in the field of glycobiology..

For information contact :
Prof. S. Paoletti
Dept. Biochemistry, Biophysics and Macromolecular Chemistry
University of Trieste, Via L. Giorgieri 1, I-34127 Trieste (Italy)
Tel. +39–40–676 36 92, fax +39–40–676 36 91
e-mail paolese@univ.trieste.it; paolese@bbcm.univ.trieste.it

The 5th International Conference

'99

AGRI-FOOD ANTIBODIES '99

SOCIETY FOR FOOD AND AGRICULTURAL IMMUNOLOGY

September 14—17th 1999

Maids Head Hotel, Norwich, UK

FIRST ANNOUNCEMENT AND CALL FOR PAPERS

Sessions will include:
- *Antibodies and Food Allergy*
- *Novel Immunochemical Approaches*
- *Antibodies and Food Functionality*
- *Antibodies and Biosensors, On-line and Hyphenated Methods*
- *Immunoassay and Contaminants*
- *Immunoassay of Phytochemicals in Food*
- *Validation and Application of Kits*
- *Antibodies and Food Adulteration and Authenticity*

There will be
- *Discussion section on issues related to production, characterisation and application of antibodies*
- *Time specifically allocated to poster sessions*

Conference secretariat
Institute of Food Research
Norwich Research Park
Colney
Norwich NR4 7UA, UK

tel. *+44–0–160 315 53 36*
Fax: *+44–0–160 350 77 23*
e-mail: *janet.pattinson@bbsrc.ac.uk*
www.ifrn.bbsrc.ac.uk/bio/big.htm

For further information about the INCOCOPERNICUS programme, please contact:

Coordinator Contact:
Pavel Rauch
Institute of Chemical Technology
Department of Biochemistry and Microbiology
Technická 3, 166 28 Prague 6, Czech Republic
Tel.: +42–2–24 35 30 76
Fax.:+42–2–311 99 90

Editor Contact:
Katarína Horáková
Ján Tkáč
Slovak Technical University
Faculty of Chemical Technology
Department of Biochemistry and Microbiology
Radlinského 9, 812 37 Bratislava, Slovakia
Tel.: +421–7–32 60 40
Fax: +421–7–39 31 98

The IncoCopernicus Contract Manager:
Mrs. Pascale Cid-Luciani
European Commision Directoriate General
Science Research and Development
Westraat 200, B–1049 Brussels, Belgium

Tel.: +32–2–296 17 06
Fax: +32–2–299 47 43
e-mail: inco-contract@dg12.cec.be

Partners e-mail:

Partner P1	rauchp@vscht.cz hocheli@vscht.cz
Partner P2	gary.wyatt@bbsrc.ac.uk gary.brett@bbsrc.ac.uk
Partner P3	horakova@chtf.stuba.sk tkac@chtf.stuba.sk
Partner P4	okennedr@ccmail.dcu.ie
Partner P5	MILCOM@login.cz
Partner P6	vumza@netlab.sk