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School of Life Sciences, Tsinghua University

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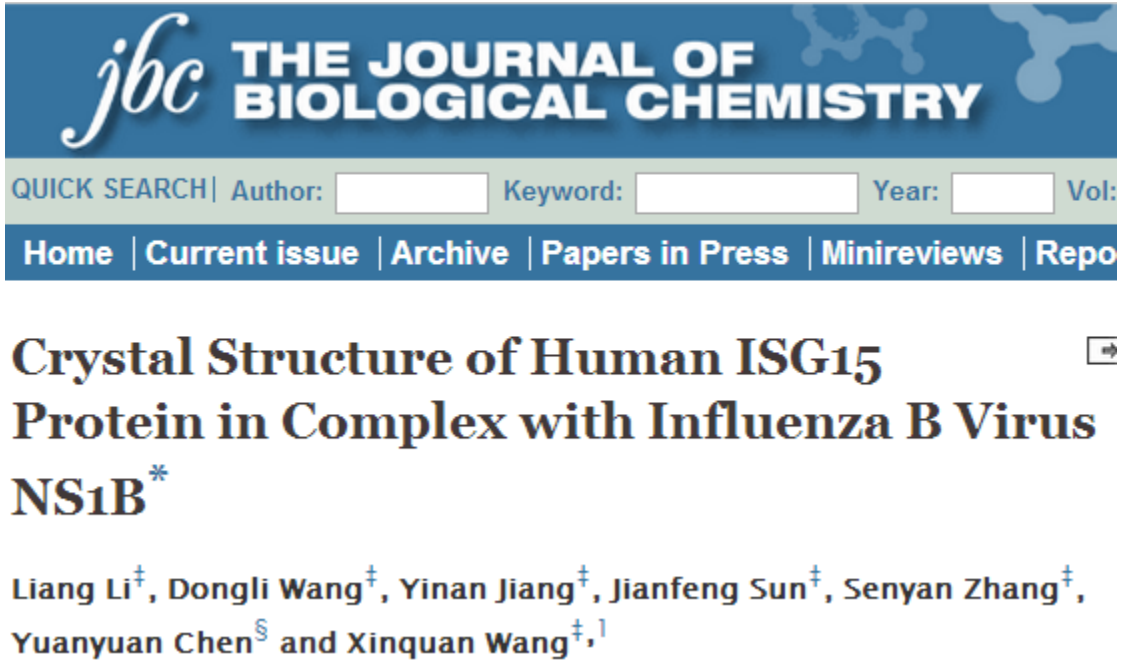

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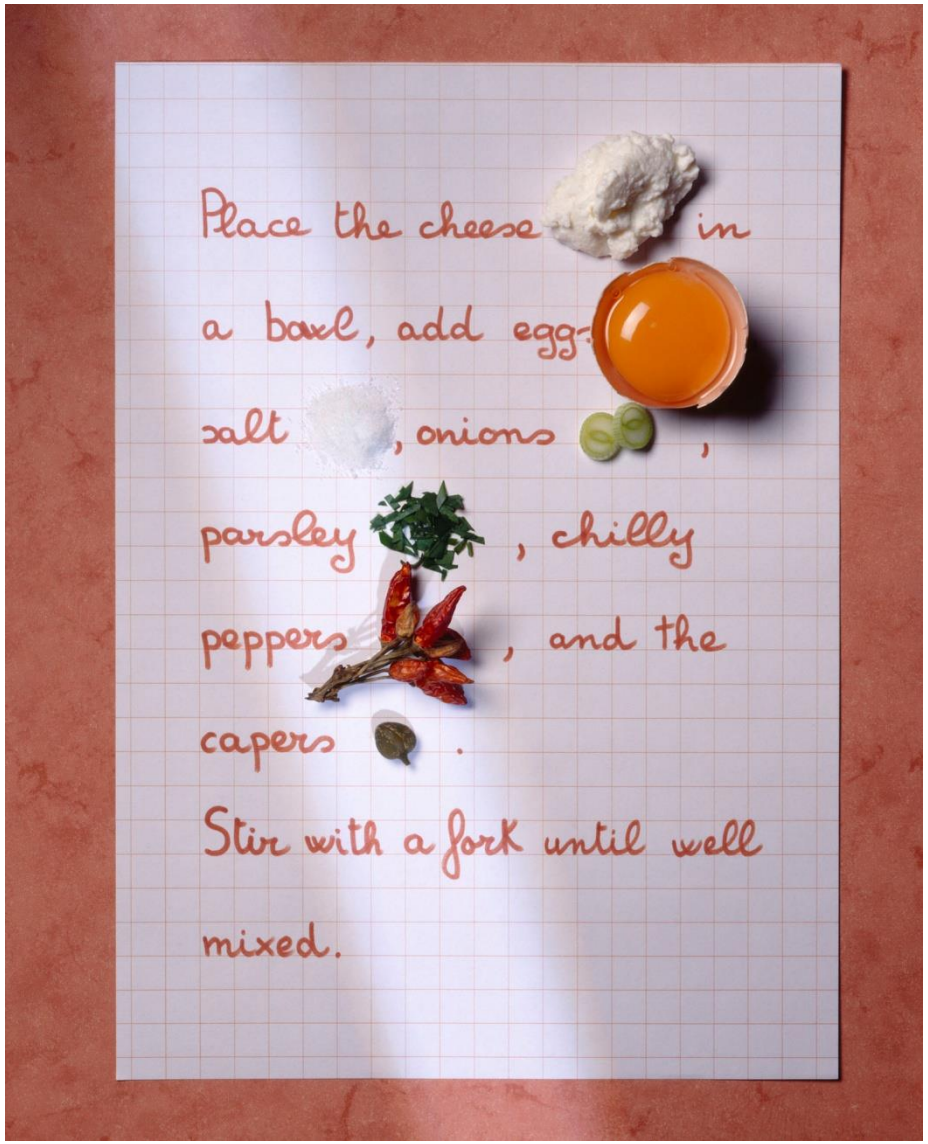

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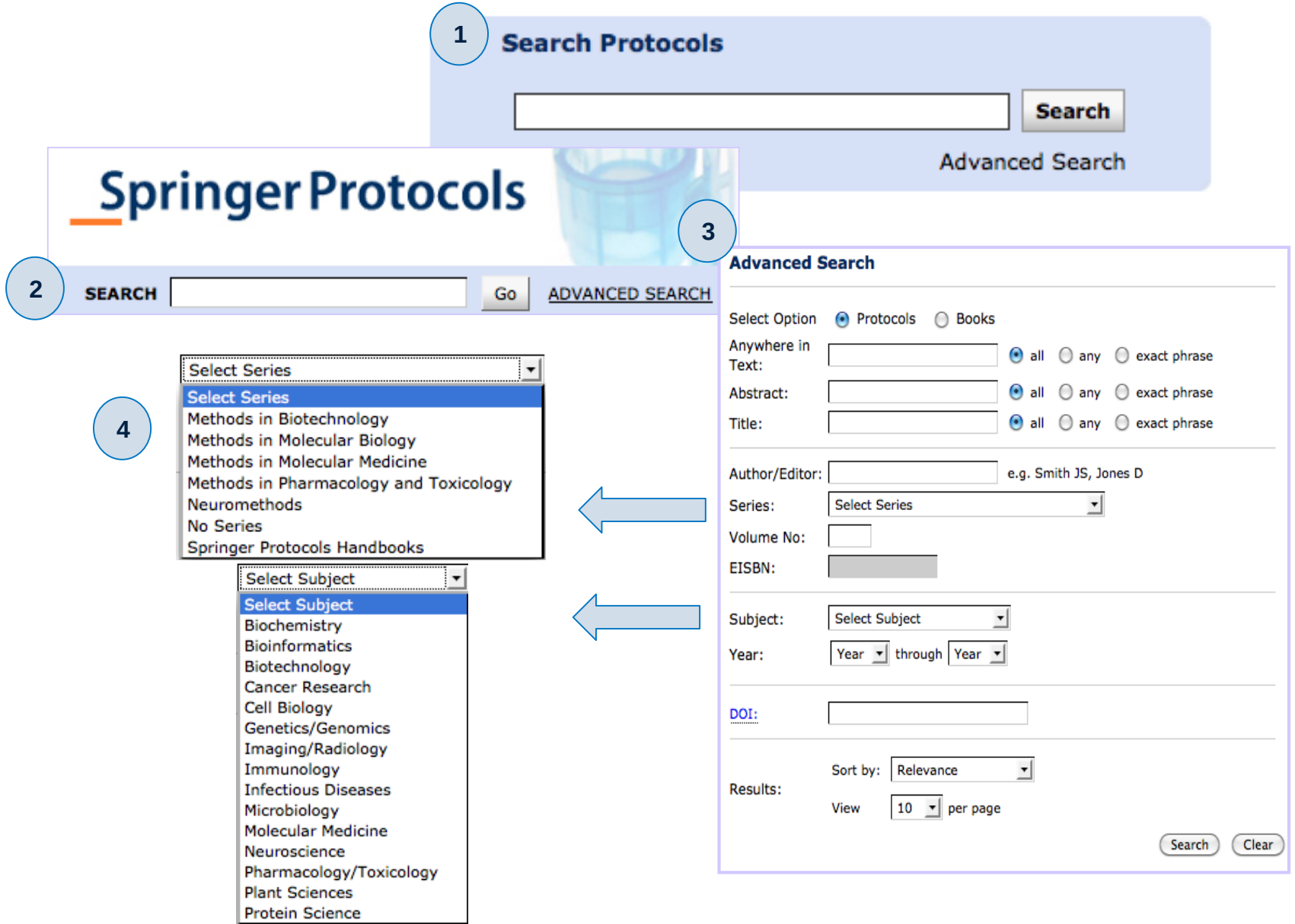

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S Assaying Peptide Translocation by the Peptide Transporter TAP

Author(s): Marlieke L. Jongsma, Jacques Neefjes

Pub. Date: Dec-03-2012; **DOI:**10.1007/978-1-62703-218-6_5

Summary: Assaying Peptide Translocation by the Peptide Transporter **TAP** MHC class I molecules display peptides at the cell surface that are mostly derived from cytosolic or nuclear proteins. Since peptide...

Abstract | Full Text | PDF (233K)

S Tandem Affinity Purification and Identification of GPCR-Associated Protein Complexes

Author(s): Avais M. Daulat, Pascal Maurice, Ralf Jockers

Pub. Date: June-15-2011; **DOI:**10.1007/978-1-61779-126-0_23

Summary: Tandem Affinity Purification and Identification of GPCR-Associated Protein Complexes The first tandem affinity purification (**TAP**) protocol was described in 1999. Originally designed...

Abstract | Full Text | PDF (141K)

S Tandem Affinity Purification of Protein Complexes from Mammalian Cells by the Strep/FLAG (SF)-TAP Tag

Author(s): Christian Johannes Gloeckner, Karsten Boldt, Annette Schumacher, Marius Ueffing

Pub. Date: Dec-01-2009; **DOI:**10.1007/978-1-60761-157-8_21

Summary: Tandem Affinity Purification of Protein Complexes from Mammalian Cells by the Strep/FLAG (SF)-**TAP** Tag Isolation and dissection

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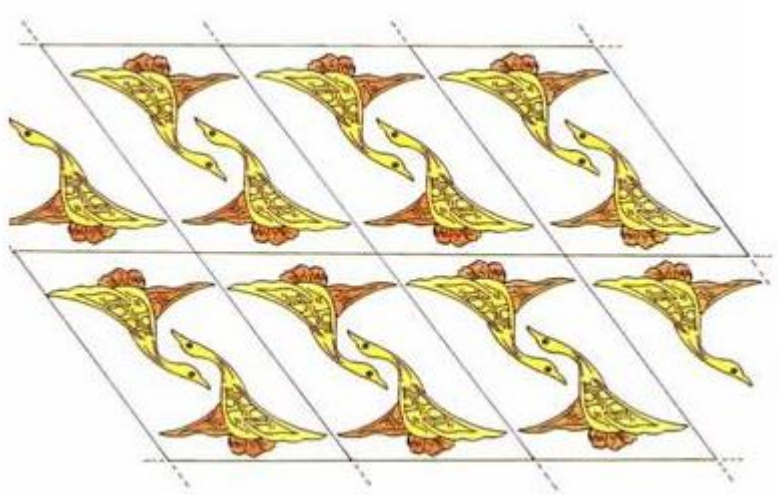

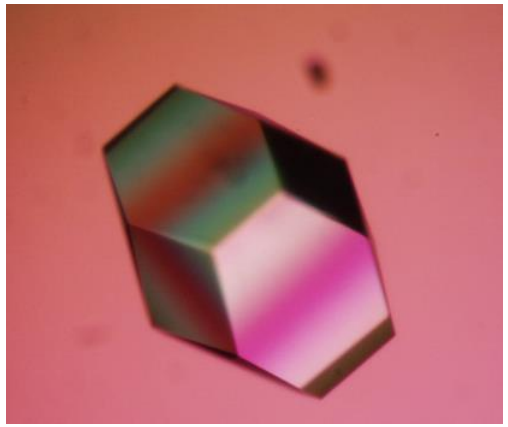

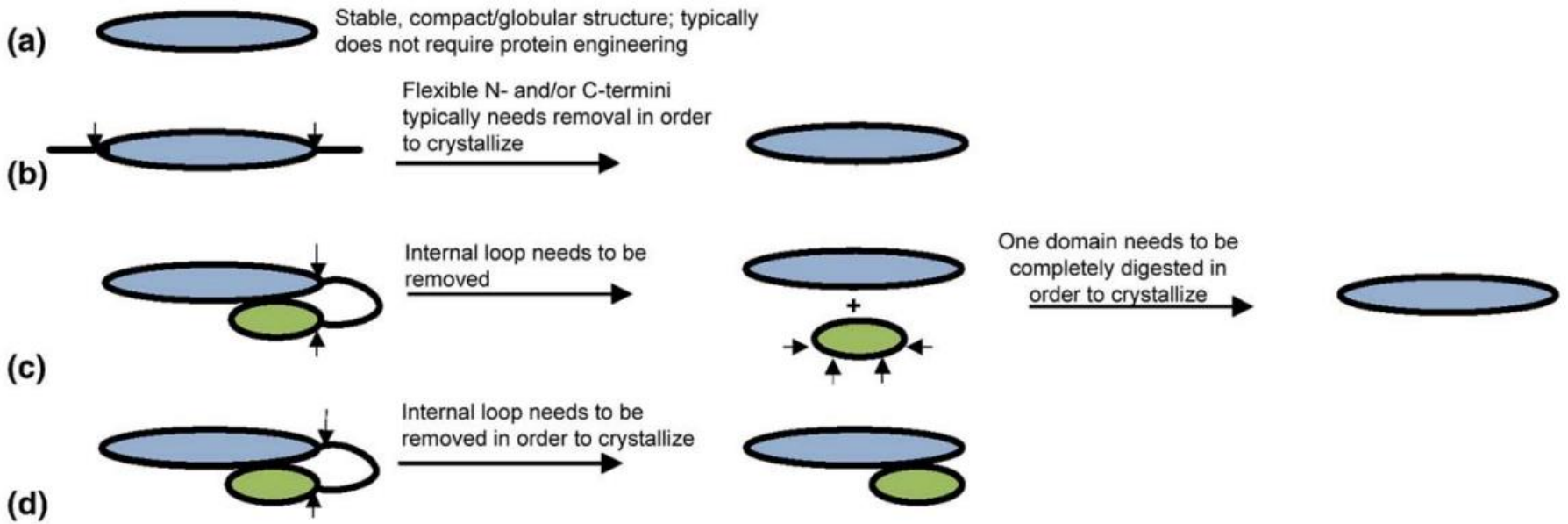

限制性酶解蛋白质去除柔性区域
利于蛋白质结晶

Gheyi T et. *J Am Soc Mass Spectro*.2010 Oct;21(10):1795-801

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DW Cleveland, SG Fischer, MW Kirschner... - Journal of Biological ..., 1977 - ASBMB
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A Fontana, G Fassina, C Vita, D Dalzoppo, M Zamai... - Biochemistry, 1986 - ACS Publications
Abstract: The preparation, characterizations, and electrochemical measurements of the terpyridine-ferrocenylalkyne spacers (tpy− CC−(fc) n− CC− tpy; tpy= terpyridyl; fc= ferrocenyl; n= 2, 3) are described. In the electrochemical measurements, the charge could ...
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H Van Tilbeurgh, P Tomme, M Claeyssens... - FEBS letters, 1986 - Elsevier
Abstract Limited proteolysis of the cellobiohydrolase I (CBH I, 65 kDa) from Trichoderma reesei by papain yields a core protein (56 kDa) which is fully active against small, soluble

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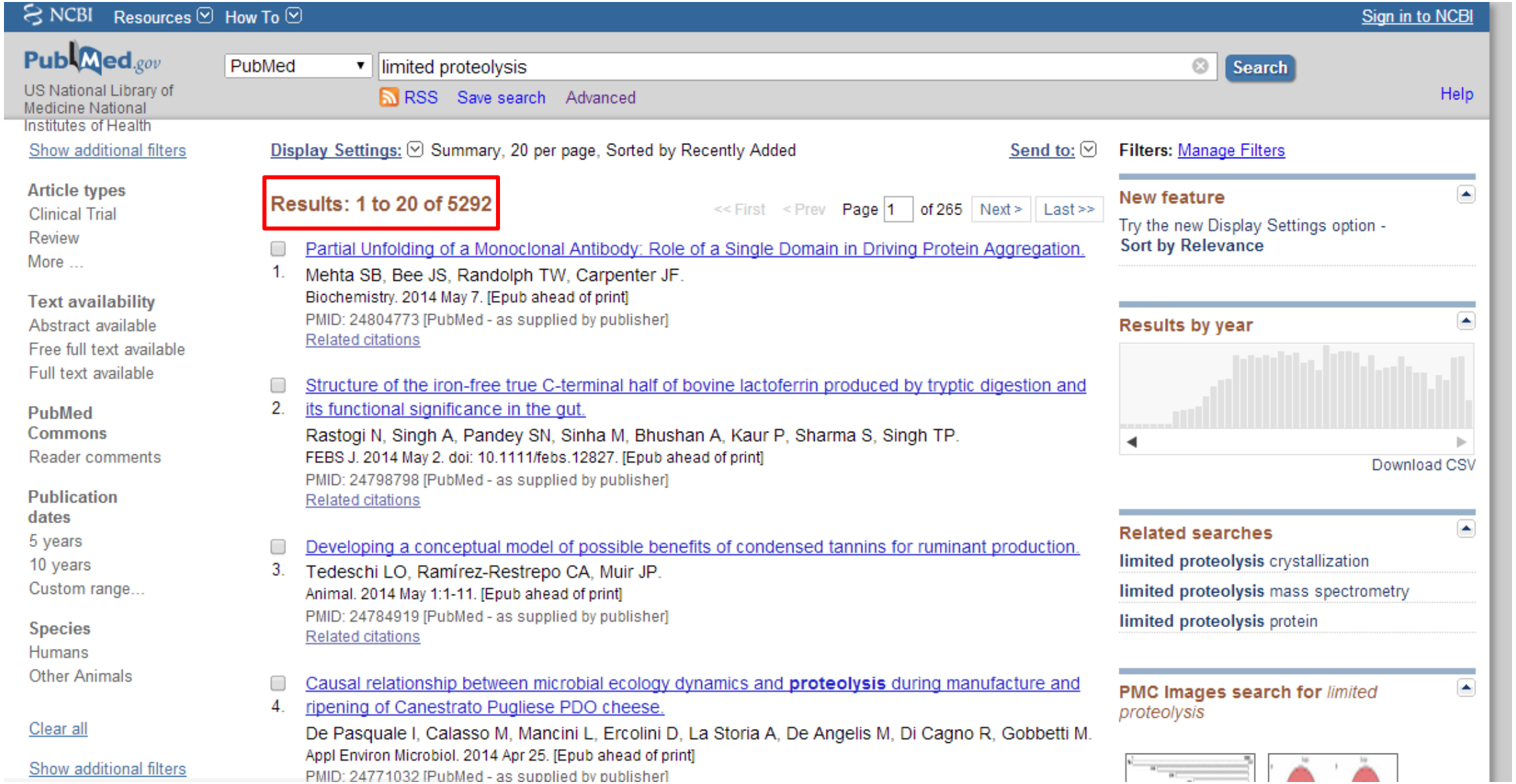

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Editor(s): Vladimir N. Uversky[1], A. Keith Dunker[2]

Affiliation(s): (1)Department of Molecular Medicine College of Medicine,University of South Florida, Tampa FL USA
(1)Institute for Biological Instrumentation,Russian Academy of Sciences, Pushchino, Moscow Region Russia
(2)Department of Biochemistry and Molecular Biology Center for Computational Biology and Bioinformatics,Indiana University, Indianapolis IN USA

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S **Immobilization of Proteins for Single-Molecule Fluorescence Resonance Energy Transfer Measurements of Conformation and Dynamics**

Author(s): Ucheor B. Choi, Keith R. Weninger, Mark E. Bowen

Pub. Date: Jul-03-2012; **DOI:** 10.1007/978-1-4614-3704-8_1; **Page Range:** 3-20

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1. Introduction | 2. Materials | 3. Methods | 4. Notes | Acknowledgments

3.1. Choice of the Protease

This work was supported by the University of Padua (ex-60%) and by the Italian Ministry of University and Research (PRIN-2007). The former lab members Paola Picotti and Marcello Zambonin have contributed a great deal to the ideas herewith expressed.

Identifying Disordered by Limited Proteolysis

Angelo Fontana, Patrizia P and Erica Frare

Vladimir N. Uversky and A.
Volume 2, Methods and Exp
DOI 10.1007/978-1-4614-3

Abstract

Limited proteolysis experiments can l
globular proteins. The approach relies
binding in an extended conformation
or local unfolding of the site of proteo
proteolysis and sites of enhanced ch
crystallographically determined *B*-fact
occurs at chain regions characterized
limited proteolysis is a simple and re
proteins, thus complementing the res
computational approaches.

Key words: Intrinsically disorder
dynamics, Proteases

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2. Tompa P (2002) Intrinsically unstructured proteins. Trends Biochem Sci 27:527–533
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5. Dunker AK, Silman I, Uversky VN, Sussman JL (2008) Function and structure of inherently disordered proteins. Curr Opin Struct Biol 18:756–764
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20. Fontana A, Zambonin M, Polverino de Laureto P, De Filippis V, Clementi A, Scaramella E (1997) Probing the conformational state of apomyoglo-

between sites of limited proteolysis and segmental mobility in thermolysin. Biochemistry 25:1847–1851

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Book Title: Intrinsically Disordered Protein A Experimental Tools

Series: Methods in Molecular Biology | **Volume:** 896 | **Pub. Date:** Jul-03-2012 | **Page Range:** 297-318 | **DOI:** 10.1007/978-1-4614-3704-8_20

实例1：限制性酶解鉴定蛋白质柔性区域

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Identifying Disordered Regions in Proteins by Limited Proteolysis

Angelo Fontana, Patrizia Polverino de Laureto, Barbara Spolaore, and Erica Frare

Abstract

Limited proteolysis experiments can be successfully used to detect sites of disorder in otherwise folded globular proteins. The approach relies on the fact that the proteolysis of a polypeptide substrate requires its binding in an extended conformation at the protease's active site and thus an enhanced backbone flexibility or local unfolding of the site of proteolytic attack. A striking correlation was found between sites of limited proteolysis and sites of enhanced chain flexibility of the polypeptide chain, this last evaluated by the crystallographically determined *B*-factor. In numerous cases, it has been shown that limited proteolysis occurs at chain regions characterized by missing electron density and thus being disordered. Therefore, limited proteolysis is a simple and reliable experimental technique that can detect sites of disorder in proteins, thus complementing the results that can be obtained by the use of other physicochemical and computational approaches.

简述实验原理

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说明方法有效

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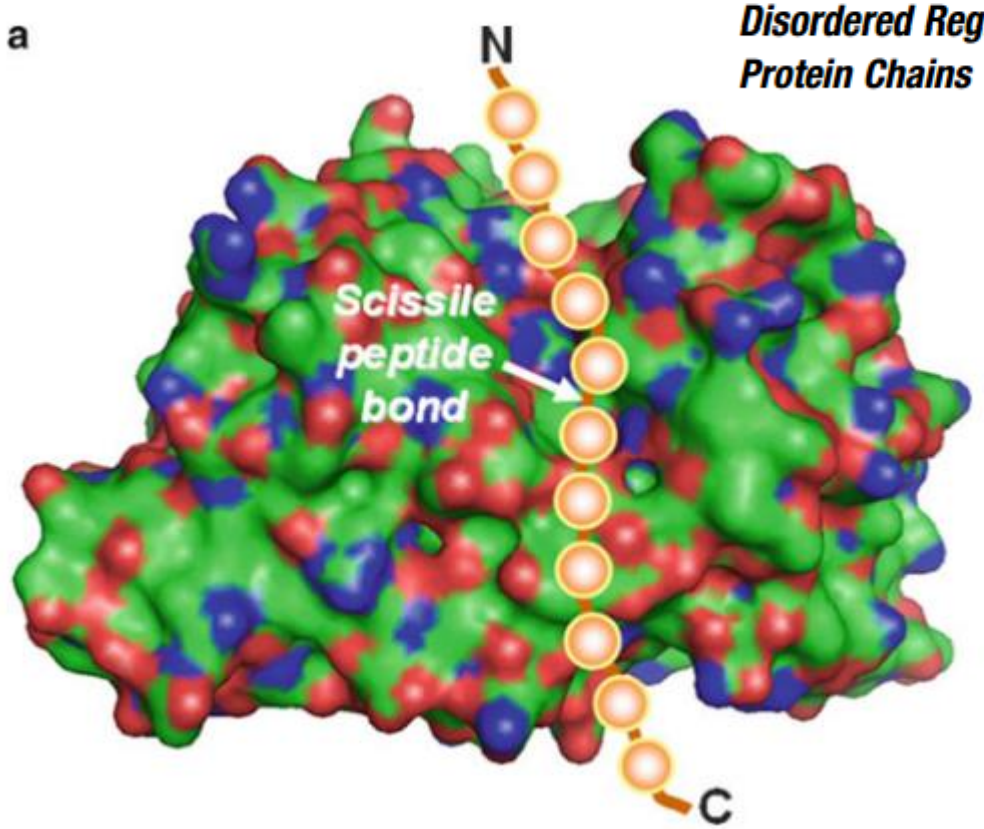
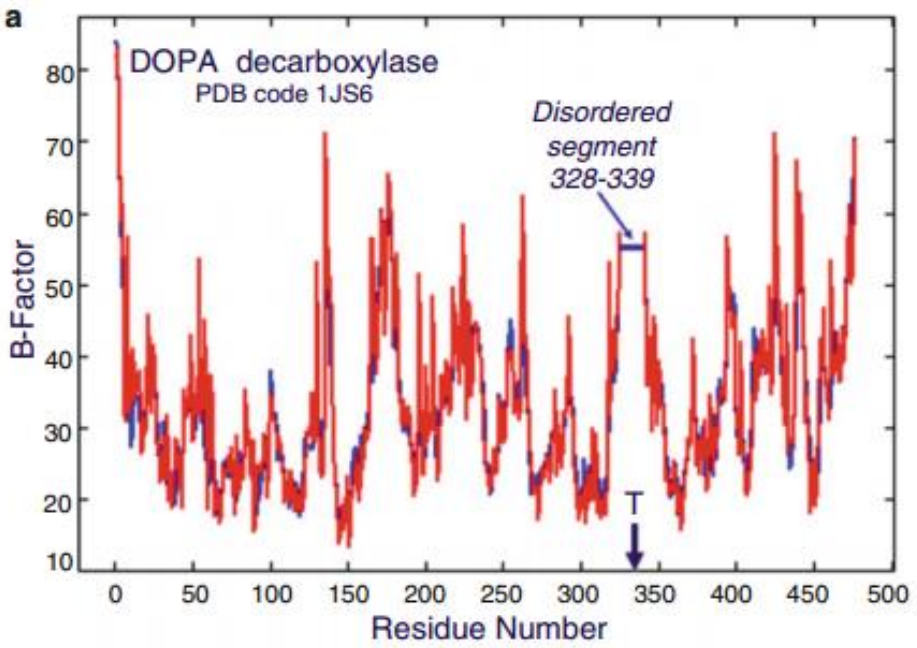
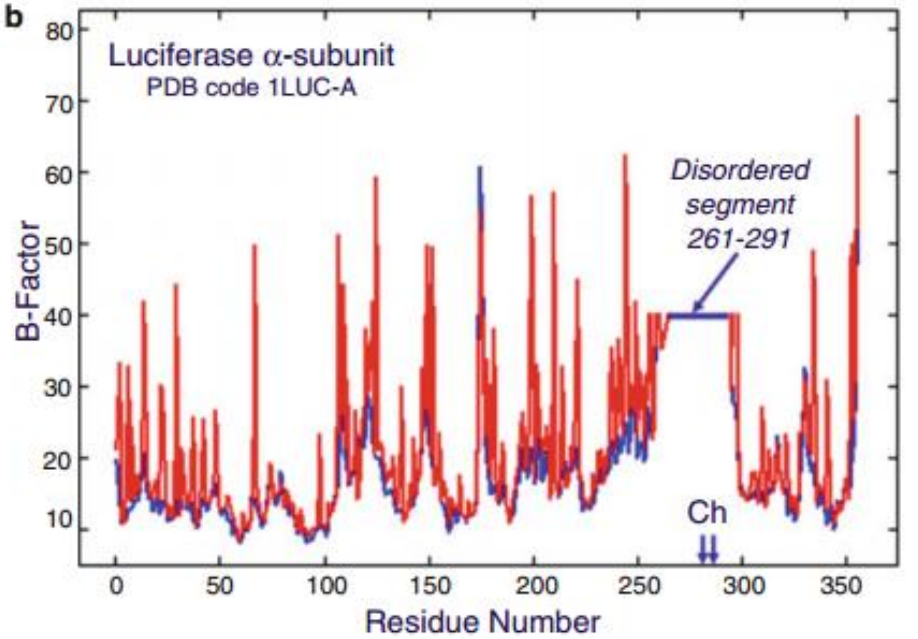
1.2. The Sites of Limited Proteolysis Occur at Disordered Regions of Protein Chains







实例1：限制性酶解鉴定蛋白质柔性区域

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2. Materials

The most used proteases listed in Table 1 can be purchased from numerous commercial sources, such as Sigma-A
sigmaaldrich.com), Promega (http://www.pro
chem (http://www.calbiochem.org), Pierce Cl
www.piercenet.com), Roche Molecular Bioche
biochem.roche.com), Biozyme Laboratories (h
com), Worthington Biochemical Corp. (http:/
biochem.com), and Protea (http://www.proteab
thermolysin from *Bacillus thermoproteolyticus*, su
subtilis, trypsin from bovine pancreas, proteinase
album, elastase from pig pancreas, and papain fro
be obtained from Sigma-Aldrich.

Table 1

Proteas							
Subtilisin (*B. subtilis*)	3.4.21.62	Ser	27,300	7.0–11.0	Nonspecific	PMSF, DFP, α_2-macroglobulin	It belongs to the nonspecific endoproteinases and thus it is most useful for detecting sites or regions of protein disorder
Thermolysin (*B. thermoproteolyticus*)	3.4.24.27	Zn	34,500	7.0–9.0	Leu, Phe, Ile, Val, Met, Ala	EDTA, citrate, phosphate, 1,10-phenathroline, phosphoramidon, α_2-macroglobulin	Rather nonspecific endoproteinase that cuts at the N-terminus of mostly hydrophobic amino acid residues. For optimal stability it may be used in the presence of 1–10 mM $CaCl_2$ and, being a thermostable enzyme, at rather high
Trypsi						PMSF, DFP,	bond. If an

提供蛋白酶的生产公司

Besides the information given in Table 1, some general properties of most commonly used proteases, details of experimental procedures, as well as suggestions for the optimal storage conditions of proteases can be found in web site of Sigma-Aldrich (http://www.sigmaaldrich.com), Promega (http://www.promega.com), Worthington Biochemical Corp. (http://www.worthington-biochem.com), and Protea (http://www.proteabio.com).

蛋白酶性质可在相应公司的网站查询

实例1：限制性酶解鉴定蛋白质柔性区域

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3. Methods

3.1. Choice of the Protease

Table 1 lists the endoproteinases with varying degrees of specificity that can be employed for limited digestion studies of proteins. Nowadays, ~20 different proteases are available from commercial sources and their purity usually is satisfactory for the specific needs of the limited proteolysis experiments. The basic premise of the limited proteolysis approach for probing protein structure implies that the proteolytic event should be dictated by the stereochemistry and flexibility of the protein substrate and not by the specificity of the attacking protease (18–22). Of course, a substrate-specific protease can cleave at a chain region only if there is a peptide bond involving a specific amino acid residue that the protease could target. Therefore, the most suitable proteases for probing protein structure disorder are those displaying broad substrate specificity, such as proteinase K, thermolysin, and subtilisin. These endopeptidases are relatively unspecific and display a moderate preference for hydrolysis at hydrophobic or neutral amino acid residues (see Table 1). The recommended approach is to perform trial experiments of proteolysis of the protein of interest utilizing several proteases of broad substrate specificity. Nevertheless, initial experiments can be conducted also utilizing proteases of more restricted specificity, such as trypsin, Glu-C protease from *Staphylococcus aureus* V8, Lys-C protease, Arg-C protease, or chymotrypsin (see Table 1). Using the latter proteases, the identification of protein fragments and thus the corresponding sites of proteolysis will be easier.

20种商业化蛋白酶

实验原理：
蛋白酶解依赖空间构象，
而非序列特异性识别

最适合的蛋白酶应有
广泛的底物特异性

预实验推荐：
使用广泛特异性的蛋白酶。
也可包括特异性更高的蛋
白酶。

实例1：限制性酶解鉴定蛋白质柔性区域

Springer

3. Methods

3.1. Choice of the Protease

Since proteases often are unstable due to autolysis, usually fresh solutions of the proteases should be employed. However, resultant variations in the exact concentrations of protease solutions prepared at different times might make it difficult to reproduce the experimental results. For this reason, it is often a good compromise simply to freeze small aliquots of protease stock solutions for future use. Protease immobilized onto solid supports (e.g., Sepharose) can be used for proteolysis experiments, since after digestion the protease can be easily removed from the proteolysis mixture by centrifugation. It is important, however, to check if the immobilized protease behaves as the one in solution, since proteolysis can be controlled also by diffusion effects of the protein substrate to the immobilized protease.

配制新鲜蛋白酶溶液，但每次配制差别导致难以重复实验

建议：配好蛋白酶溶液后分装保存

实例1：限制性酶解鉴定蛋白质柔性区域

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3.2. Controlling the Rate of Proteolytic Digestion

The conditions used for limited proteolysis studies can vary widely. In studies of limited proteolysis the enzyme:substrate (E:S) ratio employed should be specified, because proteolysis is a bimolecular reaction dependent on the concentrations of both the proteolytic enzyme and the protein substrate. E:S ratios commonly used are 1:100, but 1:1,000 or even 1:10,000 are sometimes used. Possible ways to control proteolysis are the use of a low concentration of protease, short reaction times and low temperature (21, 22). It is not easy to predict in advance the most useful experimental conditions for conducting a limited proteolysis experiment, since these actually depend on the structure, dynamics, stability/rigidity properties of the protein substrate. When optimizing reaction conditions, it is most fruitful to focus on the E:S ratio and time dependencies of the digestion course.

蛋白酶与底物的比例是实验关键。经常使用1:100

实验前难以预测反应条件

优化反应条件时，应注意蛋白酶与底物的比例，以及反应的时间

实例1：限制性酶解鉴定蛋白质柔性区域

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3.3. Inhibition and/or Inactivation of Proteases

The limited proteolysis approach implies that the hydrolytic reaction is conducted under carefully controlled conditions, since if one waits long enough extensive proteolysis of a globular protein is expected to occur, thus minimizing the utility of the approach. Of course, the initial peptide fissions are the most informative on the structural features of the protein of interest. During the manipulation of the proteolytic mixture for the isolation and analysis of "nicked" proteins or fragment species there is the risk that the mixture is exposed to denaturing solvent conditions which would render the products of initial proteolysis vulnerable to further proteolysis. It is desirable, therefore, to inhibit rapidly and irreversibly the protease at the end of the proteolytic reaction by the use of specific inhibitors (see the list of inhibitors in Table 1) or to inactivate the protease by its denaturation in acid solution or in the presence of a detergent. Metalloproteases can be rapidly inhibited by adding a metal-chelating agent (see Table 1).

应注意反应时间，否则蛋白稳定区域也开始被降解

应使用蛋白酶抑制剂终止反应

实例1：限制性酶解鉴定蛋白质柔性区域

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The easy way to stop proteolysis is to add to the proteolysis mixture trifluoroacetic acid (TFA) to a final concentration of 1.0–0.5% (by volume). An aliquot of this acid solution can be directly analyzed by reverse-phase (RP)-HPLC. It should be considered that some proteases, such as trypsin, are inhibited in acid, but recover their activity if the pH of the solution is brought to neutrality, thus causing further proteolysis. Alternatively, in order to stop proteolysis, the mixture can be added to the sample buffer used for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (65) and heated at 100°C. However, this procedure not necessarily is the most appropriate, since some proteolytic enzymes (trypsin, proteinase K, V8-protease) are not fully inactivated in the presence of SDS and there is the risk, therefore, that upon mixing the proteolytic mixture with the SDS-containing buffer proteolysis may proceed further. Therefore, proper controls should be conducted in order to check the irreversible inactivation of the protease before the SDS-PAGE analysis.

简单方法：加入TFA终止反应

备选方法：加入SDS-PAGE Buffer，并100°C加热

注意：
加对照组为实验提供参考

实例1：限制性酶解鉴定蛋白质柔性区域

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3.4. Isolation and Characterization of Protein Fragments

Since the initial cuts of a protein substrate are the most informative, the usual way to analyze the time-course of the proteolysis experiments is by SDS-PAGE. This is a method of choice, due to its simplicity, high sensitivity, high resolving power, and capability to analyze many samples in a single gel (65). Usually, 1–5 μg of a protein digest

常用方法：时间梯度酶解，SDS-PAGE检测

Protein chemistry methods combining electrophoresis or chromatography with N-terminal sequencing by the Edman technique can be used to establish the identity of the polypeptide fragments and thus to identify the nicksites along the protein chain (21). These methods can be quite labor-intensive and relatively highly demanding in terms of protein sample requirements. On the other hand, nowadays the identification of protein fragments can be made much more easily by mass spectrometry (MS) techniques (66–70). In this case, the analysis can require minute amounts of protein sample (1–10 ng, femtomoles) and can be

质谱技术发展使蛋白鉴定更加方便准确

and perform partial sequencing using tandem MS. In recent years, numerous studies have been conducted by the combined use of limited proteolysis and MS techniques (71–75).

最近几年，很多研究都将蛋白酶解与质谱分析结合

partial sequencing of peptides, thus leading to the unambiguous

实例：限制性酶解鉴定蛋白质柔性区域

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3.5. Protocols for Limited Proteolysis

A general procedure for limited proteolysis experiments on native proteins can be as follows. Prepare a stock solution of the protease in a volatile buffer such as 0.1 M ammonium bicarbonate, pH 7.8–8.5. Some proteases may slowly inactivate over time, so it is desirable to make the stock solution from the solid powder of protease as close to the start time of the experiment as possible.

配制蛋白酶溶液方法

Use a minimum volume in order to achieve high substrate concentration. Most of the commercially available proteases (see Table 1) can be used as supplied. For thermolysin, trypsin, chymotrypsin, and subtilisin the reaction mixtures should better include 1–5 mM Ca^{2+} in order to avoid autolysis. Incubate enzyme and protein substrate at a ratio of 1:100 (by weight) or, depending upon the specific protein under study, a molar ratio as low as 1:1,000–5,000 can be used. Incubate for several hours at room temperature or 37°C. However, some limited proteolysis experiments can require

提醒：一些蛋白酶溶液中需加入Ca^{2+}，防止蛋白酶自剪切

used. It is recommended to take samples at intervals (for example at 5, 10, 30, and 60 min and then after several hours), stop the reaction by acidification of these aliquots and then analyze them by SDS/PAGE or RP-HPLC.

建议在5，10，30，60分钟酶切反应时取样制备跑胶样品

实例：限制性酶解鉴定蛋白质柔性区域

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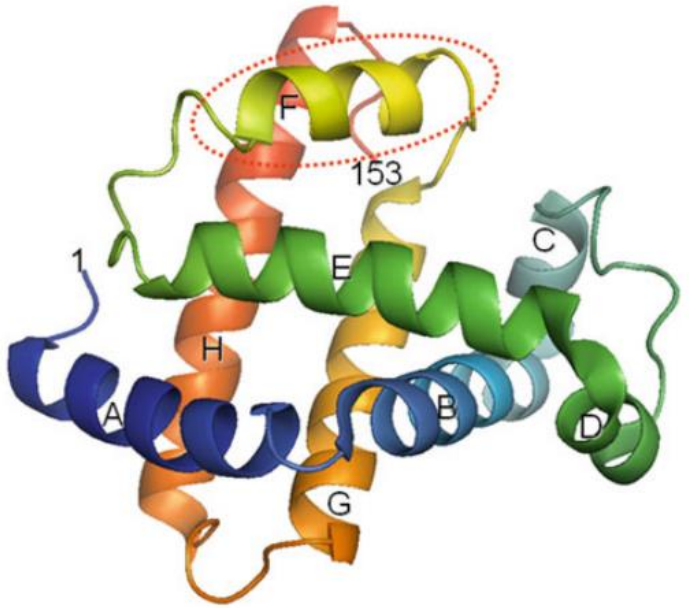
3.6. Detecting Protein Disorder in Apomyoglobin

Proteolysis of apoMb was conducted at 25°C with a variety of proteases (proteinase K, thermolysin, subtilisin, papain, elastase, chymotrypsin, V8-protease, and trypsin) (31). Limited proteolysis of apoMb was performed with the protein dissolved (0.4–0.6 mg/mL) in 20 mM Tris–HCl, 0.15 M NaCl, using an E:S ratio of 1:100 (by weight). The pH of the proteolysis mixture was 8.0 when trypsin, V8-protease, and elastase were used as proteolytic enzymes, 7.7 in proteolysis with proteinase K, and 7.5 in all other cases. The thermolysin proteolysis mixture contained also 1 mM $CaCl_2$, and with papain 1 mM cysteine.

Apomyoglobin蛋白酶解实例。

强调不同的蛋白酶在不同的PH环境下进行反应



蛋白酶解鉴定出的柔性片段与解析的结构分析符合

实例：限制性酶解鉴定蛋白质柔性区域

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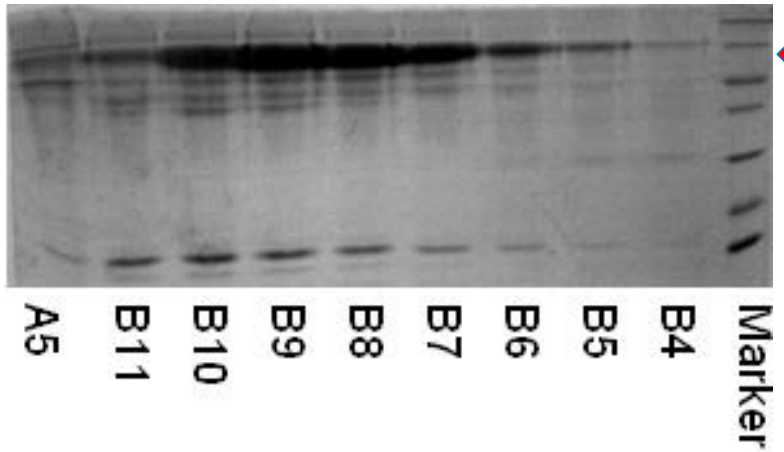
4. Notes

1. Enhanced chain flexibility (segmental mobility) is the key feature of the site(s) of limited proteolysis of globular proteins. It is amply demonstrated the sites of limited proteolysis (nicksites) in globular proteins of known 3D structure are located at chain regions which are flexible or even fully disordered, as given by the fact that these regions do not show recognizable signals in electron density maps (see Fig. 2 and Table 2).

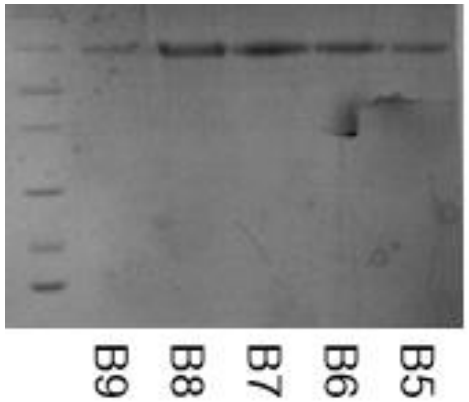
总结原理，画龙点睛

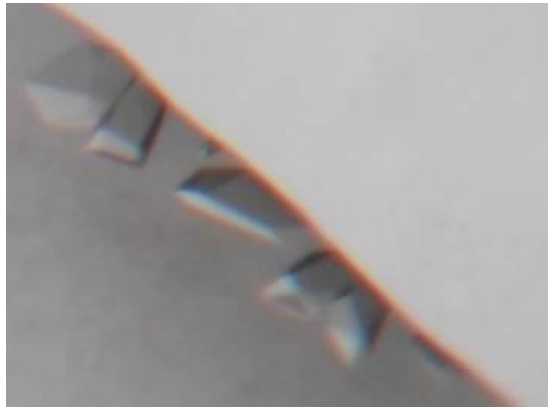
实例：限制性酶解鉴定蛋白质柔性区域

实验结果：



限制性酶解前，蛋白质条带弥散，有杂蛋白，不长蛋白质晶体





限制性酶解后，蛋白质条带单一，无杂蛋白，已长出蛋白质晶体

Chazin Lab Protocol for Limited Proteolysis

notes:

Written 10-03-02 by Melissa Stauffer

Purpose

This protocol describes the general procedure for setting up a limited proteolysis reaction. An experiment like this is useful for determining the sizes, timed order of appearance, and relative amounts of fragments produced from the protein of interest by digestion with a protease. From the results, you may be able to infer the presence of stable subdomains. It is also possible to obtain evidence of binding of one protein to another by running two identical sets of proteolysis experiments, one with the putative target, and one without. If the rate of digestion of the protein is slowed by the addition of the putative target, one can conclude that they interact with each other.

The basic procedure involves exchanging the protein of interest into an appropriate buffer system and diluting to a desired volume. A protease is then added to start the reaction, and aliquots of the reaction are removed at specific time points. The aliquots are then boiled to inactivate the protease and the results are analyzed by SDS-PAGE.

Variables to consider

1. Amount of protein
2. Amount of protease
3. Type of protease
4. Reaction volume
5. Time increments
6. Gel sample size
7. Staining method

Comments and suggestions corresponding to each of these variables are found below:

与网络上protocol比较

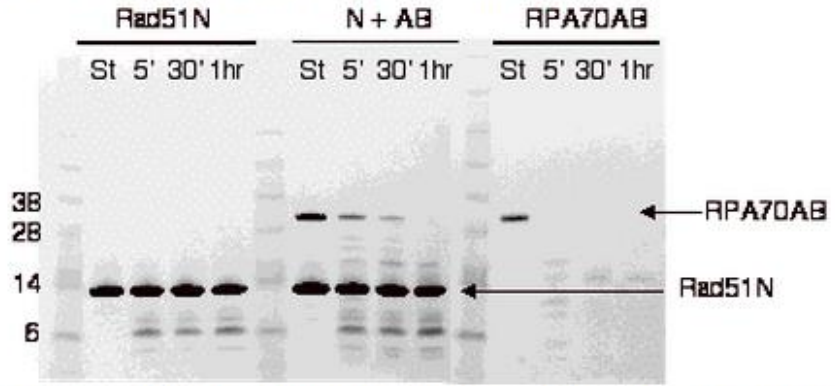
Considerations

1. Determine the amount of protein you want to expend on the experiment. This will automatically determine the type of gel staining you use. In my experience, the following chart is a good estimate:

   ug protein » ng protease » breakdown in hours » silver stain;
   ug-mg protein » ug protease » breakdown in minutes » coomassie stain.
2. The amount of protease should be about 1000x less than the amount of protein in mass units. This quantity can be adjusted so the rate of proteolysis fits the desired time frame. Protease solutions can be made in water, but they may lose activity over time, so make the stock solution from solid powder as close to the start time of the experiment as possible.
3. Choose several different types of protease for the first try. We routinely use chymotrypsin, trypsin, and proteinase K to probe a wide range of specificity.
4. The reaction volume should be designed to provide the appropriate number of gel sample aliquots. For example, 80 mL will give eight 10-mL gel samples. Be sure that each aliquot will contain an observable amount of protein for the staining method you choose.
5. Time increments can be chosen based on what is convenient. As stated above, the amount of protease can be adjusted so the reaction goes faster or slower. The total reaction time can be as short as a single hour or as long as a whole day (or more), depending on how much detail you need

Example

The following gels were obtained from digestion of ~400 ug protein with ~20 ng proteinase K in 20 mM Tris, pH 8.0, 1 mM BME. Total reaction volume was 80 uL, and 10 uL gel samples were removed. The gels were stained with Coomassie.



As you can see, the middle gel shows that the addition of Rad51N protein reduces the rate of degradation of RPA70AB, thus implying a physical interaction between them.

实例1：限制性酶解鉴定蛋白质柔性区域

实例2：串联亲和纯化法鉴定相互作用蛋白

实例2：串联亲和纯化法鉴定相互作用蛋白

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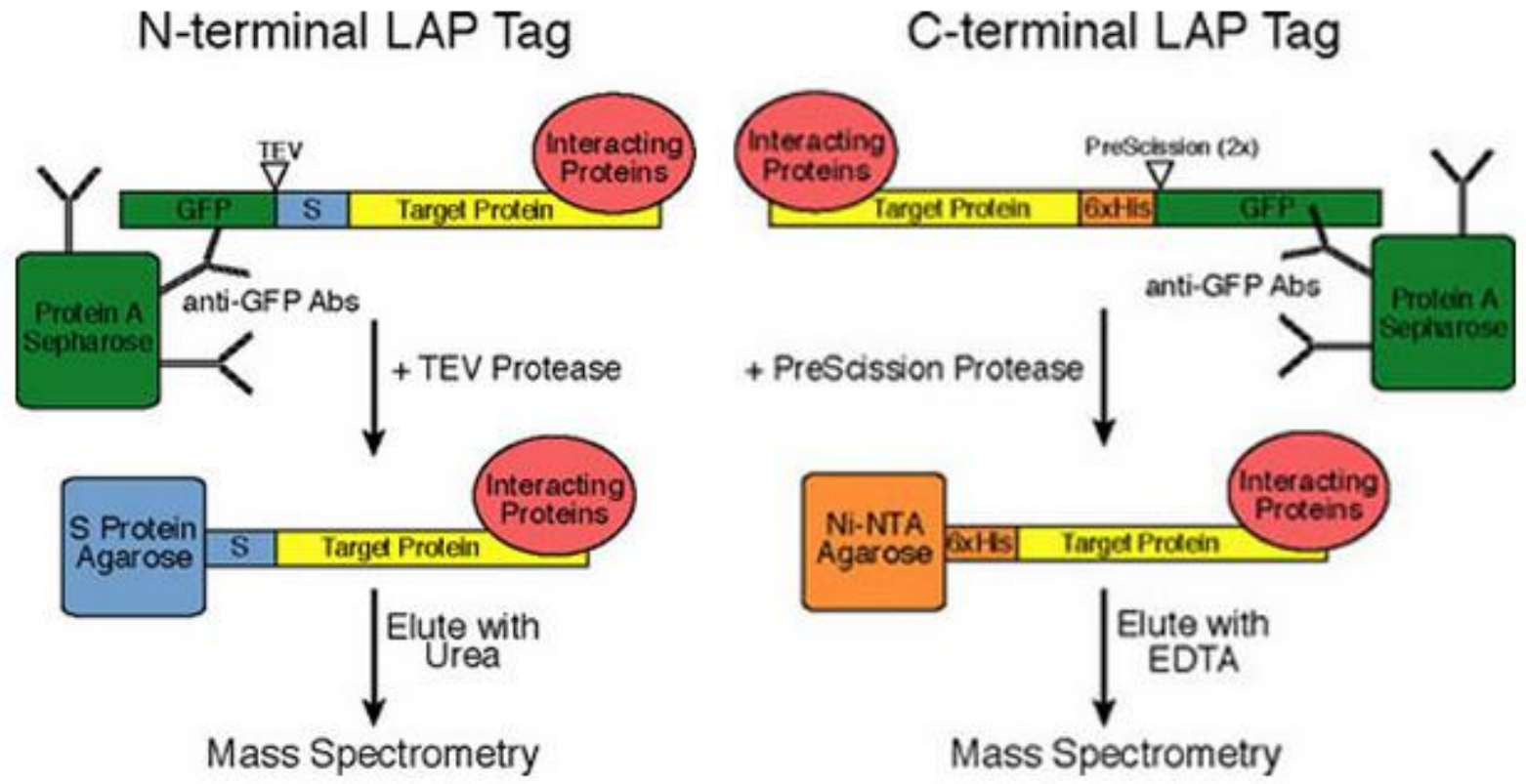
串联亲和纯化（tandem affinity purification TAP）是一种研究体内蛋白质相互作用的技术，经过两步特异性亲和纯化，可快速得到生理条件下与靶蛋白质存在真实相互作用的蛋白质。



Cheeseman lab

实例2：串联亲和纯化法鉴定相互作用蛋白

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实例2：串联亲和纯化法鉴定相互作用蛋白

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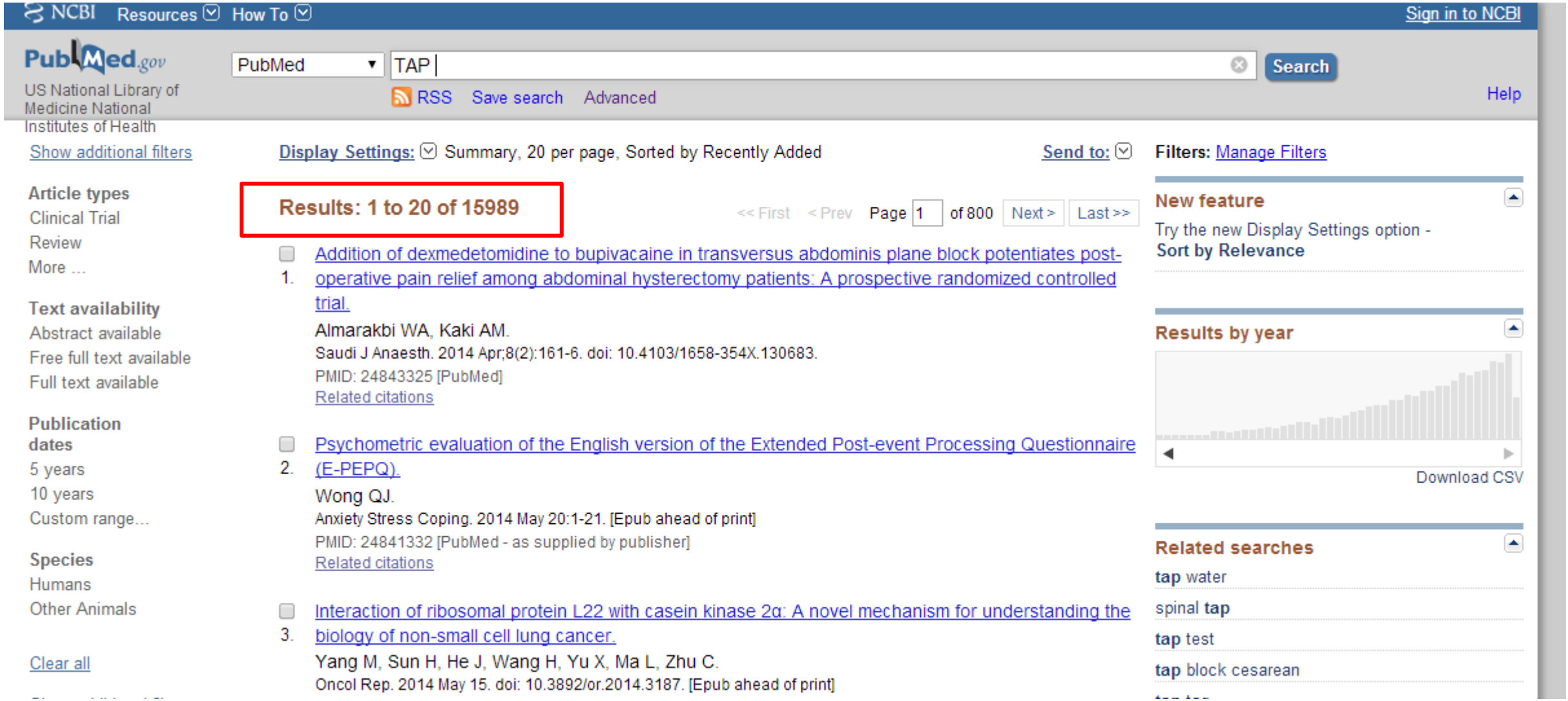

实例2：串联亲和纯化法鉴定相互作用蛋白

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Author(s): Marlieke L. Jongsma, Jacques Neefjes

Pub. Date: Dec-03-2012; **DOI:**10.1007/978-1-62703-218-6_5

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Author(s): Avais M. Daulat, Pascal Maurice, Ralf Jockers

Pub. Date: June-15-2011; **DOI:**10.1007/978-1-61779-126-0_23

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Author(s): Christian Johannes Gloeckner, Karsten Boldt, Annette Schumacher, Marius Ueffing

Pub. Date: Dec-01-2009; **DOI:**10.1007/978-1-60761-157-8_21

Summary: Tandem Affinity Purification of Protein Complexes from Mammalian Cells by the Strep/FLAG (SF)-TAP Tag Isolation and dissection of native multiprotein complexes is a central theme in functional...

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Tandem Affinity Purification of Protein Complexes from Mammalian Cells by the Strep/FLAG (SF)-TAP Tag

Christian Johannes Gloeckner, Karsten Boldt, Annette Schumacher, and Marius Ueffing

Summary

Isolation and dissection of native multiprotein complexes is a central theme in functional genomics. The development of the tandem affinity purification (TAP) tag has enabled efficient and large-scale purification of native protein complexes. The SF-TAP tag, a modified version of the TAP tag, allows a fast and straightforward purification of protein complexes from mammalian cells. It consists of a tandem Strep-tag II and a FLAG epitope (SF-TAP). The SF-TAP tag allows a native elution of protein complexes without proteolytic cleavage needed in the original TAP procedure. Besides the SF-TAP protocol, the principal idea of a pathway mapping by subsequent tagging of copurified proteins is demonstrated for the interactome of the MAPKKK Raf.

简述实验原理

提供实验证据

说明方法有效

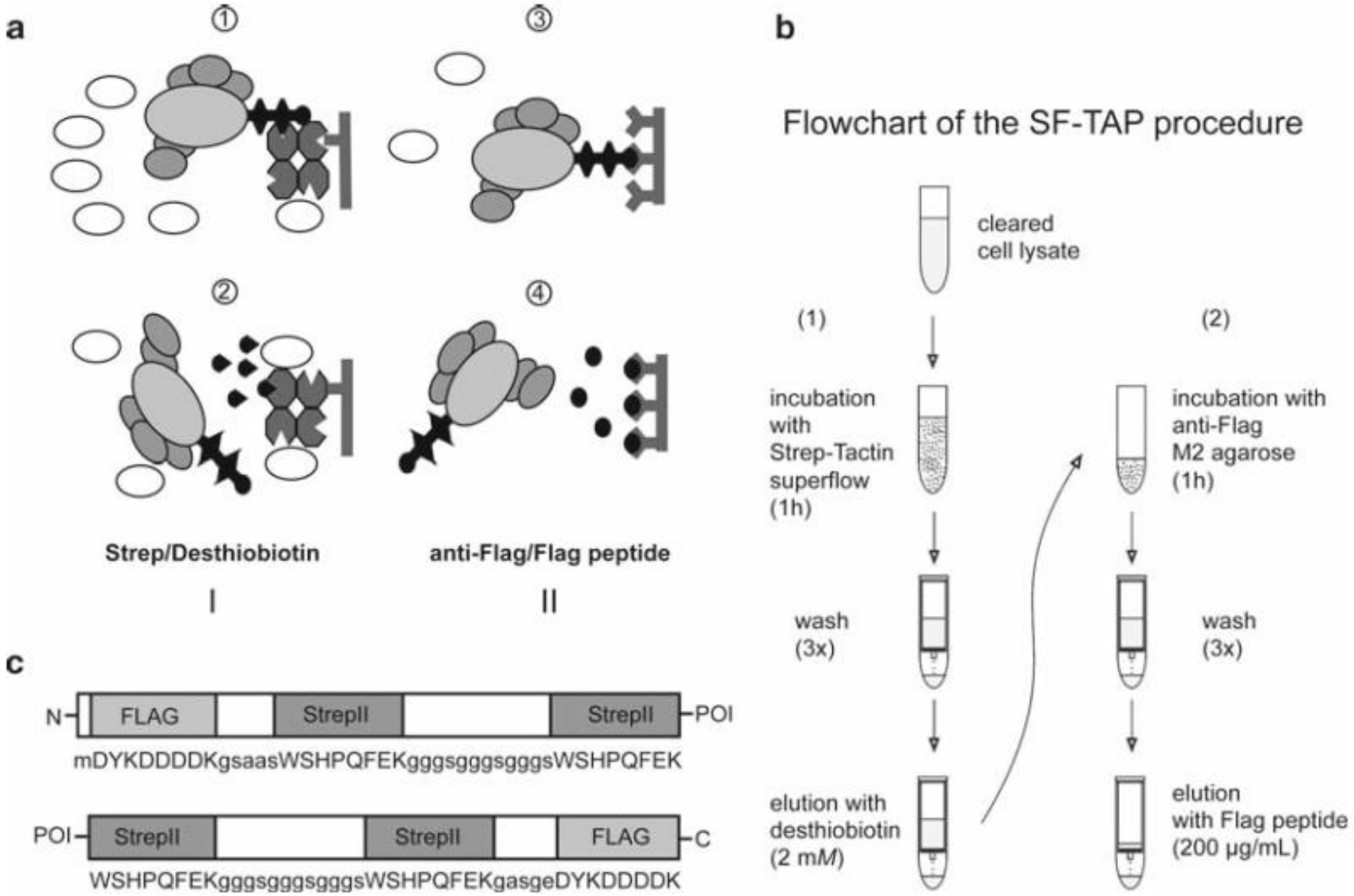
1. Introduction

affinity purification step. Recently, we developed a novel TAP combination (SF-TAP), which omits the proteolytic cleavage due to the combination of medium affinity tags, which can be eluted by competition *(8)*. Additionally, the selection for rather small tags resulted in a fourfold size reduction of the SF-TAP tag (5 kDa) when compared with the original TAP tag (21 kDa). This TAP combination consists of a tandem Strep-tag II *(9, 10)* and a FLAG tag (SF-TAP). Desthiobiotin is used for the elution

实验原理：不需酶切，蛋白标签小，不会影响目标蛋白空间结构

including the tag sequence is shown in **Fig. 1**. Owing to omission of the proteolytic step, the overall time for a TAP could be reduced to 2½ h when compared with the original TAP protocol.

因不需酶切，实验操作时间仅需2.5小时


a
①
③
②
④
Strep/Desthiobiotin
anti-Flag/Flag peptide
I
II
b
Flowchart of the SF-TAP procedure
cleared
cell lysate
(1)
(2)
incubation
with
Strep-Tactin
superflow
(1h)
incubation with
anti-Flag
M2 agarose
(1h)
wash
(3x)
wash
(3x)
elution with
desthiobiotin
(2 mM)
elution
with Flag peptide
(200 µg/mL)
c
N
FLAG
StrepII
StrepII
POI
mDYKDDDDKgsaasWSHPQFEKgggsgggsgggsWSHPQFEK
POI
StrepII
StrepII
FLAG
C
WSHPQFEKgggsgggsgggsWSHPQFEKgasgeDYKDDDDK

实例2：串联亲和纯化法鉴定相互作用蛋白

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2. Materials

2.1. Gateway Cloning

1. *Escherichia coli ccd* B-survival T1 phage resistant ($T1^R$) strain (Invitrogen) for amplification of donor (pDONR) and destination (pDEST/N-SF-TAP, pDEST/C-SF-TAP) plasmids.

2.2. Cell Culture of HEK293 Cells

2.3. Transfection of HEK293 Cell and Generation of Cell Lines Stably Expressing the SF-TAP Fusion Protein

2.4. Cell Lysis and SF-TAP Purification

1. Cell scraper (Sarstedt).
2. Millex GP 0.22 µm syringe driven filter units (Millipore).
3. Microspin columns (GE Healthcare).

2.5. Chloroform Methanol Precipitation

1. Two milliliter sample tube (Eppendorf).
2. Chlorophorm (Merck).
3. Methanol (Merck).

2.6. Alkylation and Tryptic Digestion

1. RapiGest stock solution: 2% RapiGest (Waters) in water.
2. Fifty millimolar ammoniumbicarbonate: dissolve 0.39 g of ammoniumbicarbonate (Sigma-Aldrich) in 100 mL of water

实例2：串联亲和纯化法鉴定相互作用蛋白

Springer

3. Methods

3.1. Cloning of SF-TAP Expression Constructs by Gateway Cloning

The Gateway cloning system (Invitrogen) is based on site-specific recombination mediated by the λ integrase family of recombinases *(14)*. It allows fast and easy cloning of inserts, a prerequisite for the generation of larger numbers of expression vectors. The Gateway system consists of two classes of vectors, the donor and the

克隆方法

3.2. Transfection of HEK293 Cells and Generation of Cell Lines Stably Expressing the SF-TAP Fusion Protein

For pilot experiments, the SF-TAP approach can be tested in HEK293 cells transiently expressing the bait protein. There are two major advantages of using HEK293 cells: (1) The transfection efficiency is usually very high. (2) They are fast growing and thereby produce high amounts of protein (10–15 mg total protein per 14 cm dish corresponding to ~7 × 10^7 HEK293 cells). For the transfection of one 14 cm dish, 1–4 µg of plas-

转染及制备稳定细胞系

3.3. SF-TAP Purification Protocol

A flowchart of the SF-TAP purification procedure is shown in **Fig. 1b**.

1. Remove the medium from the plates.

纯化步骤

实例2：串联亲和纯化法鉴定相互作用蛋白

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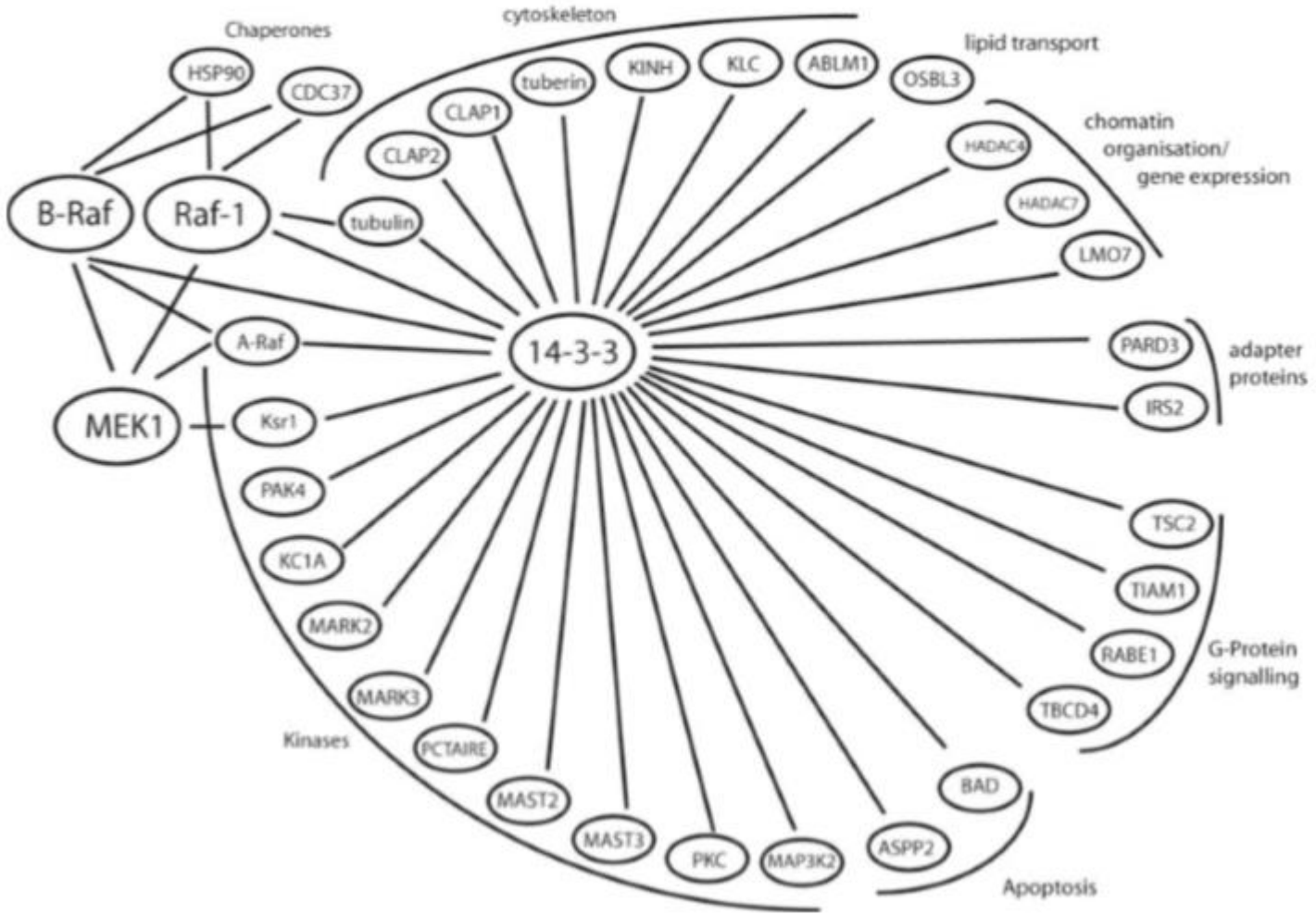
3.4. Chloroform–Methanol Precipitat (According to Wess and Flügge [15])

3.5. Preparation of Samples for an LC-MS/MS Approa (Alkylation and Try Digest)

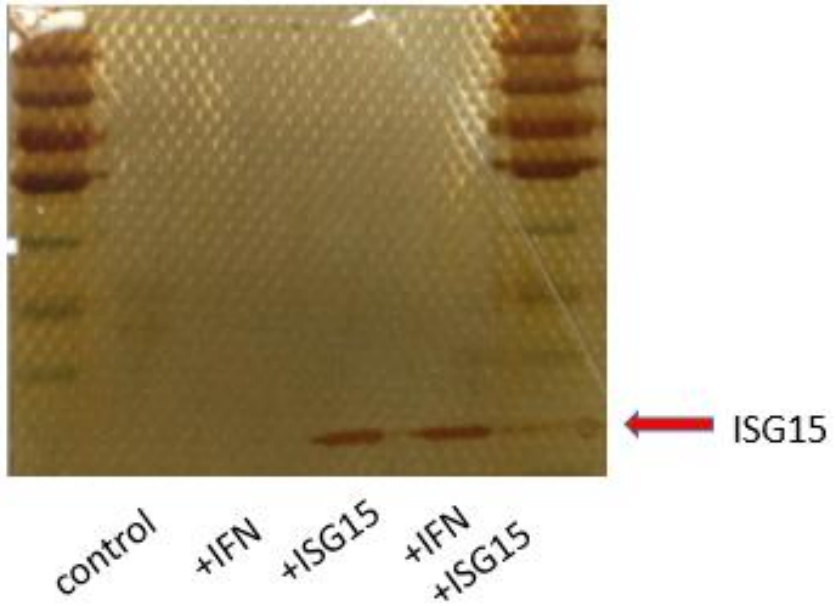
3.6. Pathway Walk for Raf-1 and B-Ra



制备蛋白样品供质谱检测

实例说明

实例2：串联亲和纯化法鉴定相互作用蛋白



实验结果：已鉴定出一个与目标靶蛋白相互作用的蛋白质，正与其它实验室合作研究此蛋白质复合物发挥功能的分子机理。

Springer Protocols是科学研究的得力帮手，良师益友，值得拥有！

谢谢！