YAXINBIO

Scientific questions of peptide mapping analysis of protein/antibody
蛋白及抗体肽图分析中的科学问题

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Regulations 法 规 要 求

❖ 1999年ICH Q6B”肽图分析”描述

d) Peptide map

Selective fragmentation of the product into discrete peptides is performed using suitable enzymes or chemicals and the resulting peptide fragments are analyzed by HPLC or other appropriate analytical procedure. The peptide fragments should be identified to the extent possible using techniques such as amino acid compositional analysis, N-terminal sequencing, or mass spectrometry. Peptide mapping of the drug substance or drug product using an appropriately validated procedure is a method that is frequently used to confirm desired product structure for lot release purposes.

- 肽片段可以采用氨基酸组成分析、N末端测序或质谱方法进行鉴别。
- 经过验证的原液和成品肽图方法可用作确证预期产品结构的批放行指标。

Regulations 法 规 要 求

❖ 2013年WHO rDNA指南”肽图分析”描述

a) Peptide map

Selective fragmentation of the product into discrete peptides is performed by using suitable enzymes or chemicals. The resulting peptide fragments are analysed by high-performance liquid chromatography (HPLC) or other appropriate analytical procedures.

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The peptide fragments should be identified as far as possible using appropriate techniques such as mass spectrometry (MS) methods (e.g. electrospray ionization MS, matrix-assisted laser-desorption ionization time-of-flight MS). The use of MS/MS coupling should also be considered as it could reveal more detailed sequence information about the analysed peptide fragment. If one fragmentation method does not deliver the complete amino acid sequence, the use of an orthogonal enzyme or chemical cleavage method can increase the sequence coverage. The correct formation of the disulfide bridges may be characterized by the use of peptide mapping under reducing and non-reducing conditions.

- 肽片段可以采用质谱方法进行鉴别（例如：ESI-MS，MALDI-TOF-MS）。
- 可采用MS/MS方法用来揭示肽片段的详细信息。

肽图分析面临的问题

ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
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JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Validation of a peptide mapping method for a therapeutic monoclonal antibody: what could we possibly learn about a method we have run 100 times?☆

Jacob Bongers [a,*], John J. Cummings [a], Mary Beth Ebert [a], M. Marcia Federici [c], Linden Gledhill [a], Dipti Gulati [a], George M. Hilliard [a], Brian H. Jones [a], Kwan R. Lee [d], Jacek Mozdzanowski [a], Michael Naimoli [b], Sudhir Burman [a]

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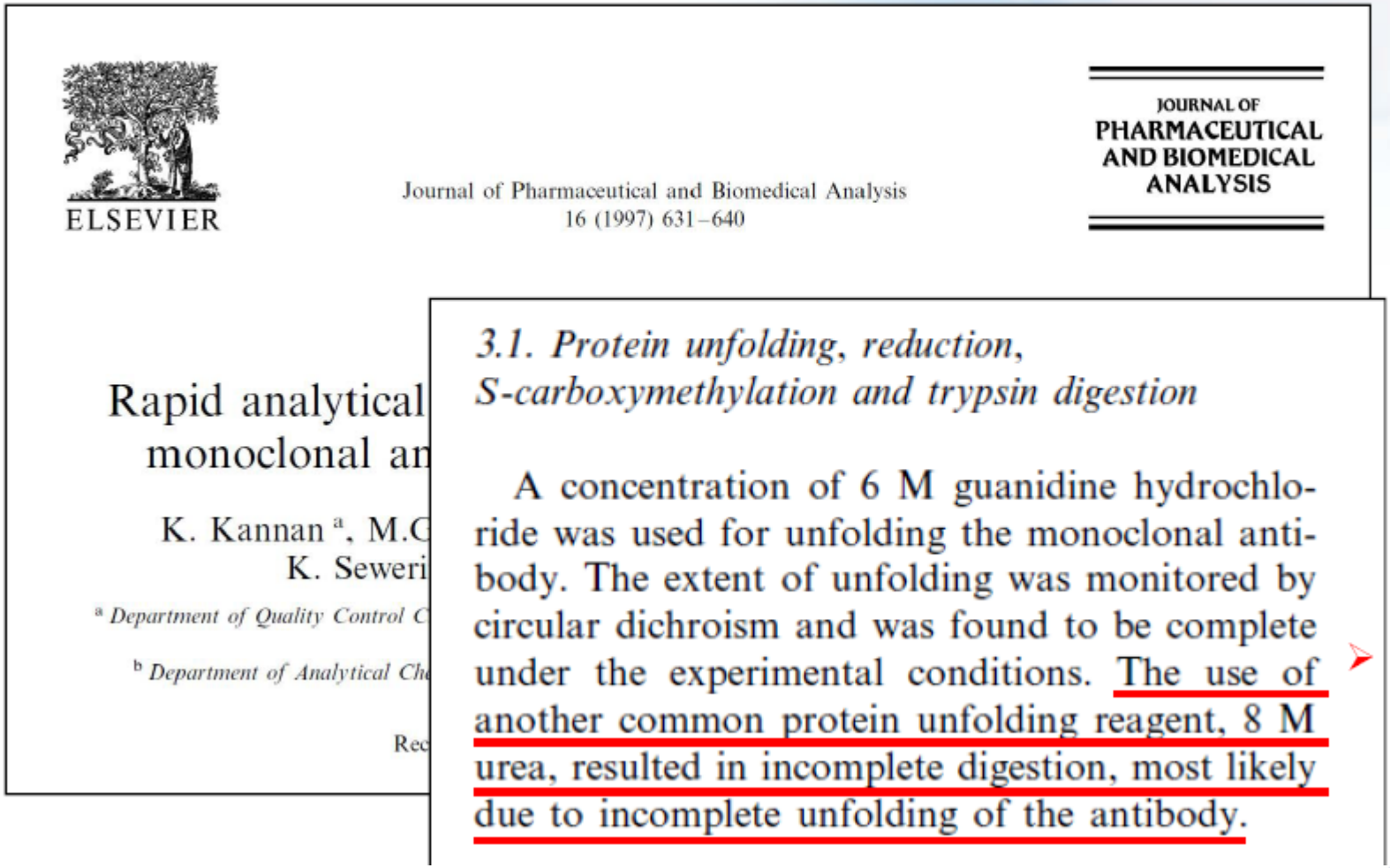
Accepted 2 August 1999

前处理—变性、还原要点

❖ 盐酸胍或尿素的选择：



ELSEVIER

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JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Rapid analytical
monoclonal an

K. Kannan [a], M.C
K. Seweri

[a] Department of Quality Control C

[b] Department of Analytical Ch

Rec

3.1. Protein unfolding, reduction, S-carboxymethylation and trypsin digestion

A concentration of 6 M guanidine hydrochloride was used for unfolding the monoclonal antibody. The extent of unfolding was monitored by circular dichroism and was found to be complete under the experimental conditions. The use of another common protein unfolding reagent, 8 M urea, resulted in incomplete digestion, most likely due to incomplete unfolding of the antibody.

8M尿素不能使抗体完全变性，导致酶切不完全

前处理—酶切体系要点

❖ 蛋白酶切体系的换液方式：

BioProcess TECHNICAL

Solution-Phase Sample Preparation Approach for Peptide Mapping of Biological Therapeutics

Vajira Nanayakkara, William E. Werner, and Thomas Theriault

Table 2: Summary of data for protein 1, comparing the PD10 column buffer exchange, dialysis, and ultrafiltration methods

	PD10	Dialysis	Ultrafiltration
Sequence coverage by AA residues (%)	75	80	94
H chain C — terminal Lys clipped	Yes	Yes	Yes
H chain Asn 34, 99, and 349 glycosylation	Weak signal	Weak signal	Yes
H chain N — terminal deamidation	Not found	Weak signal	Yes
Salt adduct ions	Yes	Yes	None or trace
Data quality and reproducibility	Poor	Poor	Excellent
Sample processing time per sample	36 hours	36 hours	12 hours

前处理思考要点汇总

◆抗体/蛋白 变性是否充分？
酶切时抗体/蛋白是否仍保持**一致**的非折叠状态，对于肽图分析的**重现性**至关重要。

◆同时重视工具酶trypsin的作用条件和稳定性条件，是另一个重要的前提。

Rituximab	*USP Medicine Compendium Final Authorized Version 1.0 (2012)*
Trastuzumab	*USP Medicine Compendium Final Authorized Version 1.0 (2013)*
Bevacizumab	*USP Medicine Compendium Final Authorized Version 1.0 (2014)*

USP—peptide mapping

Published on *USP Medicines Compendium* (https://mc.usp.org)

Rituximab

Final Authorized Version 1.0

还原条件下，
酶切>80%

- **B. Peptide Mapping**

Use a chromatographic system. (Proceed as directed in *Biotechnology Derived Articles—Peptide Mapping* <1055>.)

Analyze the material to be tested by a chromatographic technique capable of resolving peptides generated from a Trypsin digest. The digest is carried out under reducing conditions which provides NLT 80% digestion. The test procedure used provides a minimum of 90% coverage of the protein sequence.

Standard solution: Digest and dilute a portion of USP Rituximab RS in an appropriate diluent.

Sample solution: Digest and dilute a quantity of Rituximab in an appropriate diluent to obtain a nominal concentration of Rituximab similar to that of the *Standard solution*.

Control solution: Digest and dilute a portion of an appropriate control (non-Rituximab monoclonal antibody) in an appropriate diluent to obtain a nominal concentration of the control that is similar to that of *Standard solution*. [Note—The digests described in the *Standard solution*, *Sample solution*, and *Control solution* are conducted at the same time, using the same stock and concentration of reagents.]

USP—peptide mapping

IDENTIFICATION

- **A. Peptide Mapping**

Solution A: 0.1% Trifluoroacetic acid in water

Solution B: 0.1% Trifluoroacetic acid in acetonitrile

Solution C: 0.5 M dithiothreitol in water

Solution D: 0.5 M iodoacetamide in water

Solution E: 0.25 M tris buffer in water. Adjust with dilute hydrochloric acid to a pH of 7.5.

Solution F: 6 M guanidine hydrochloride and 1 mM EDTA in *Solution E* (denaturing buffer)

Solution G: 0.1 M tris buffer in water. Adjust with dilute hydrochloric acid to a pH of 7.8.

Solution H: 2 M urea in *Solution G* (digest buffer)

Solution I: 0.05 M acetic acid in water

Solution J: 1 mg/mL of trypsin in *Solution I*

Solution K: 10 mg/mL of USP Rituximab RS in water

Standard stock solution 1: Mix 100 µL of *Solution K*, 400 µL of *Solution F*, and 10 µL of *Solution C*, and incubate at 37° for 30 min. Add 24 µL of *Solution D* and incubate at room temperature for an additional 30 min in dark. Add 10 µL of *Solution C* and mix well.

Standard stock solution 2: Wash the PD-10 Sephadex G-25 column with 20 mL of water and equilibrate with 35–40 mL of *Solution H*. Load *Standard stock solution 1* on the column, and elute using *Solution H* in volumes of 700 µL each. Collect 6 independent fractions. Measure the absorbance of each fraction at 280 nm against *Solution H*. The fraction having an absorbance between 1.3 and 2.0 is used for digestion. [Note—If the absorbance of the fraction is more than 2.0, dilute it using *Solution H* to get an absorbance of 2.0.]

Standard solution: Mix well 50 µL of *Standard stock solution 2* and 2 µL of *Solution J* and incubate for 18–20 h at room temperature. Add 1 µL of trifluoroacetic acid and store the mixture at 4°.

6M 盐酸胍变性

2M 尿素中酶切变性

G25脱盐柱

室温酶切
18–20h

USP—peptide mapping

Questions:

1) **Different buffer for denaturing (6M GuHCl) and for digest (2M urea)**
2) **Mode for buffer changing: G25 column**
3) **Digestion time: 18-20h in room temperature**

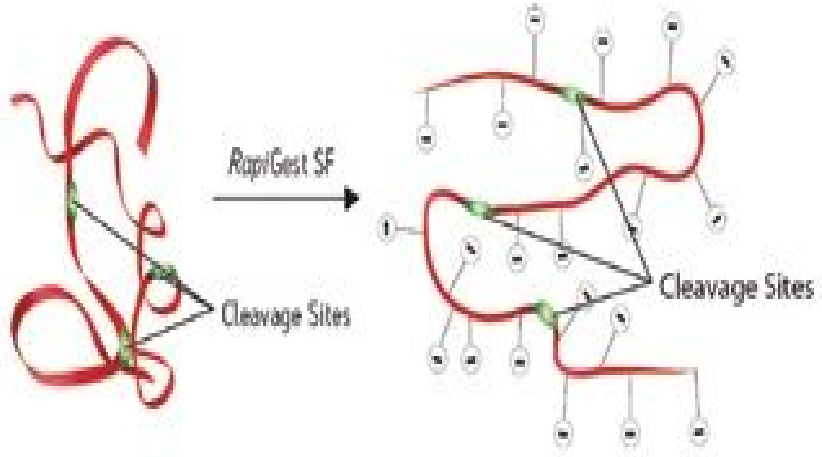
MS鉴定与UV放行

1) MS鉴定：比如你有100ug蛋白，酶切了50ug，足以在肽图上找到所有的肽段和后修饰，那么剩下的50ug没切开也没事，所以看到waters、国外药企发的文章一般都是酶切4-5小时，酶切过久你也看到会增加翻译后修饰的比例，对于鉴定来说是不利的。

2) UV放行，不看后修饰也不看覆盖率，要的是UV图谱与药典标准图谱的比对。这是要保证100ug蛋白全部切开的。所以经常看到药企里，还有USP做UV放行监控都是过夜酶切甚至24小时酶切，而且是超滤或者过柱子换液后酶切的。

Sample pretreatment for peptide mapping

Rapigest SF surfactant from Waters



Similar as SDS, but more effective, and good activity for trypsin in Rapigest

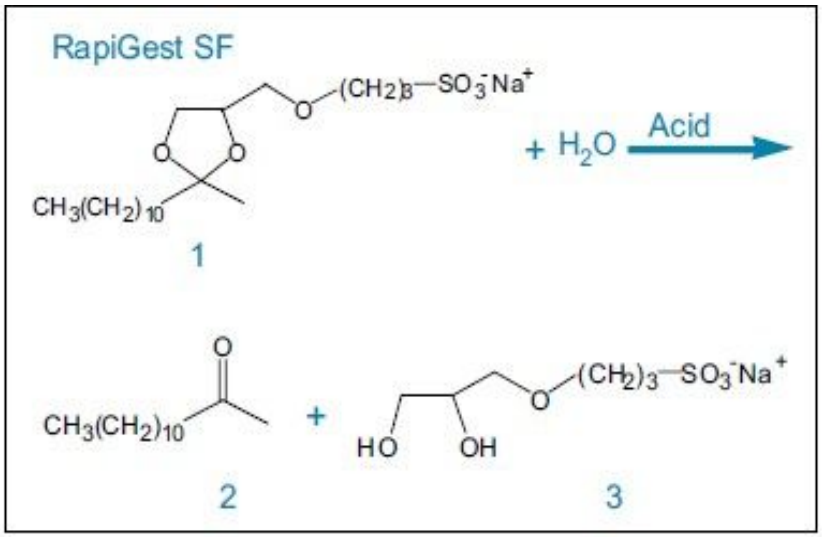


图2 RapiGest SF (1)在酸性溶液中降解为(2)和(3)，在pH 2为2时，$t_{1/2}$为7.6分钟

Rapigest SF surfactant from Waters

表1 在选择的变性剂存在的情况下检测胰蛋白酶活性

胰蛋白酶溶液[A]	胰蛋白酶活性[B](%)	胰蛋白酶溶液[A]	胰蛋白酶活性[B](%)
无添加剂	100	50% 甲醇	31
0.1%RAPIGEST	100	50% 乙腈	92
0.5% RAPIGEST	87	1M 尿素	97
0.1% SDS	20	2 M尿素	83
0.5 SDS	1	0.5 M 盐酸胍	21
0.1 RAPIGEST/0.1% SDS	58	1 M盐酸胍	8

A 将0.5 μg胰蛋白酶加入1 mL 50 mM的重碳酸胺溶液中, Ph 7.9, 含有0.2 mM的BAEE

B △BAEE在253 nm下的吸收值(5分钟内的斜率)

启示：

找到一种条件，在此条件下，trypsin保持活性而目的蛋白溶解变性。

Sample pretreatment for peptide mapping

Rapigest SF surfactant from Waters

Suggested procedure for in-solution digestions

1. Suspend lyophilized Rapigest SF powder in 1mL of 50mM NH4HCO3 to give 0.1%(w/v).
2. Suspend protein pellet in
3. Add DTT to 5mM
4. Heat the sample at 60℃ for 30 min
5. Cool the sample
6. Add IAA to 15mM and place the sample in dark 30min
7. Add enzyme for digestion (1:100 to 1:20, w/w)
8. Incubate at 37 fro 1hr to overnight depending upon protein hydrophobicity

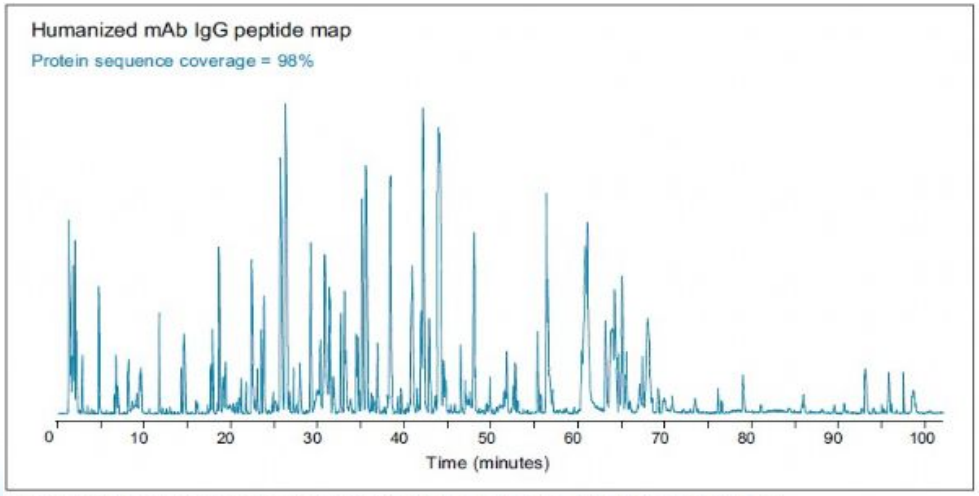


图4 胰蛋白酶消解人化单抗的LC/MS 分析。样品制备的复杂性显著降低。无需消化后清洁。总进样量为10 pmol 胰蛋白酶单抗。

Enzymes for peptide mapping

Sequencing grade trypsin:

TPCK-trypsin

Promega

Sigma

Recombinant trypsin for peptide map

YaxinBio

Roche

TPCK-Trypsin

PRODUCT NAME	comments	Content of chymotrypsin in Trypsin
USP-trypsin	USP standard	<5%
Trypsin (sample)	Trypsin sample before TPCK treated	1.8%
TPCK-trypsin	Trypsin after TPCK treated	0.56%

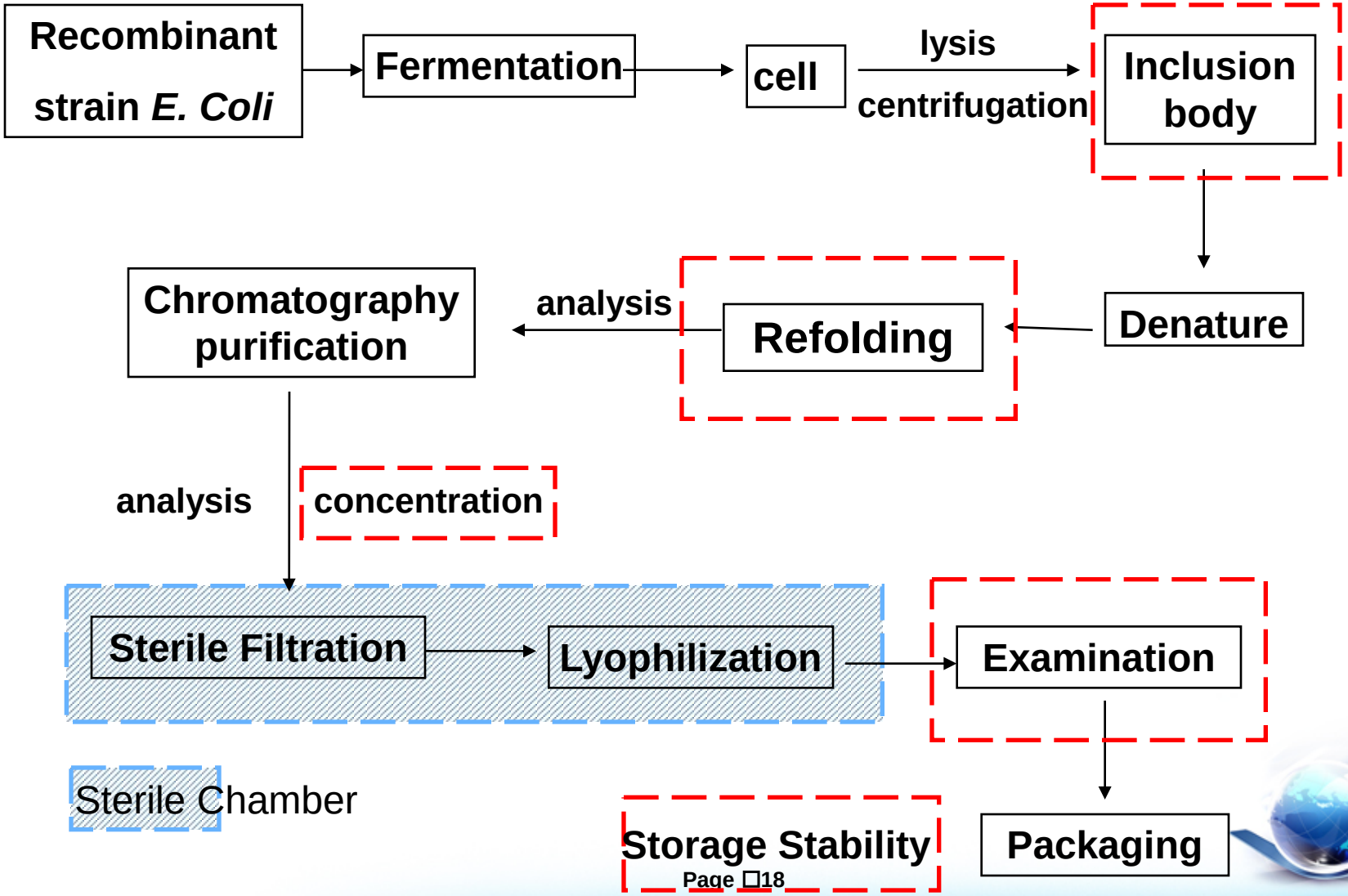
There is chymotrypsin activity in TPCK-treated trypsin.

Recombinant Trypsin

No any other enzymes activity.
So, it is unnecessary for TPCK treated.
不含任何其他杂酶活性
不需TPCK处理

High specific activity:高特异性

RECOMBINANT TRYPSIN 重组胰蛋白酶



USP R-trypsin

美国药典重组胰蛋白酶标准

39(5) In-Process Revision: <89> ENZYMES USED AS ANCILLARY MATERIALS... Page 1 of 7

BRIEFING

〈89〉 **Enzymes Used As Ancillary Material in Pharmaceutical Manufacturing.** This new general test chapter provides analytical procedures to aid in the assessment of quality for enzymes that are used in biopharmaceutical manufacturing. Examples include trypsin, collagenase, pepsin, and papain. This chapter does not discuss the applications of these enzymes but rather focuses on tests to assess the qualities as process materials. Further, the chapter does not provide ways to limit the enzymes in the final medicinal product. The first enzyme discussed in the chapter is recombinant trypsin. Other enzymes will be added in subsequent revisions of the chapter.

制药过程中的辅料—酶

为保证生物制药过程中酶的质量，提供酶质量的分析方法，包括胰蛋白酶、胶原酶、胃蛋白酶和木瓜蛋白酶。

第一个讨论的是重组胰蛋白酶。

The activity of recombinant trypsin is determined using a chromogenic peptide substrate carbobenzoxy-valyl-glycyl-arginine-4-nitril-anilide acetate. The liquid chromatographic procedure in the test for *Purity* is based on analyses performed with the YMC-Pack ODS-A brand of L1 column. The retention time for the main peak is 12–17 min. A new Reference Standard, USP rTrypsin RS, is proposed for use during assessment of the system suitability for the proposed *Assay* and test for *Purity*.

(BIO: E. Chang.)
Correspondence Number—C126264

Comment deadline: November 30, 2013

HPLC的方法分析trypsin的纯度

YAXINBIO

Comparation of trypsin

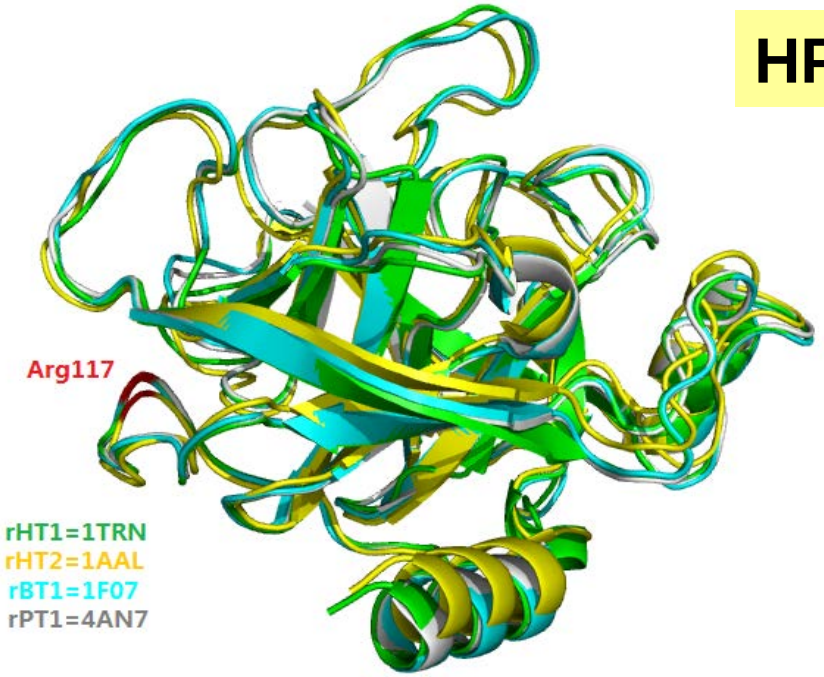
Trypsin

Test/Method	Specification 1:2500 in USP/CP	Specification 1:250
Appearance	White powder	Yellowish or brown
solubility	Soluble	Soluble overnight
Microbial limits	-	none
Staphylococcus aureus	Negative	none
Pseudomonas aeruginosa	Negative	none
Salmonella species	Negative	none
Loss on drying	NMT 5.0%	none
Residue on ignition	NMT 2.5%	none
Limit of Chymotrypsin	NMT 5.0%	none
Activity	NLT 2,500 USP units/mg	NLT 250 USP units/mg

Recombinant trypsin in USP2014

Test/Method	Specification
Solubility	soluble
Bioburden	NMT 100 CFU/ml
Specific ctivity (USP u/mg pro)	NLT 3800
Purity (RP-HPLC)	NLT 70% β-trysin, NMT 20% α-trypsin

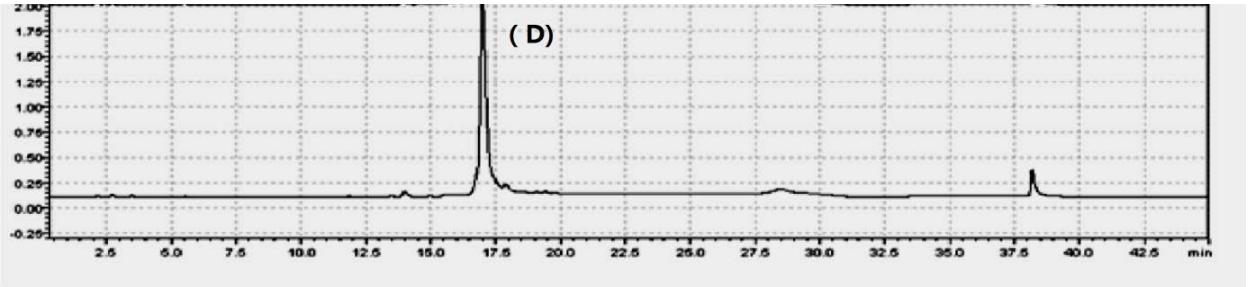
USP中，两种胰蛋白酶的标准的主要不同点



HPLC 方法检测胰蛋白酶的纯度

The retention time for the main peak for r-trypsin is 12-17min.
主峰的保留时间为： 12-17min

Retention time: NLT 1.0min between two peaks of α-trypsin and β-trypsin.
α –胰蛋白酶和 β –胰蛋白酶的保留时间相差不小于1.0min.



NLT 70% for the peak area of β-trypsin and NMT 20% for the peak area of α-trypsin.
按照峰面积计算： β –胰蛋白酶>70%，α –胰蛋白酶<20%。

Stability of rTrypsin

不同浓度重组胰蛋白酶在pH7.8 50mM NH4HC03, 37℃ 下的稳定性

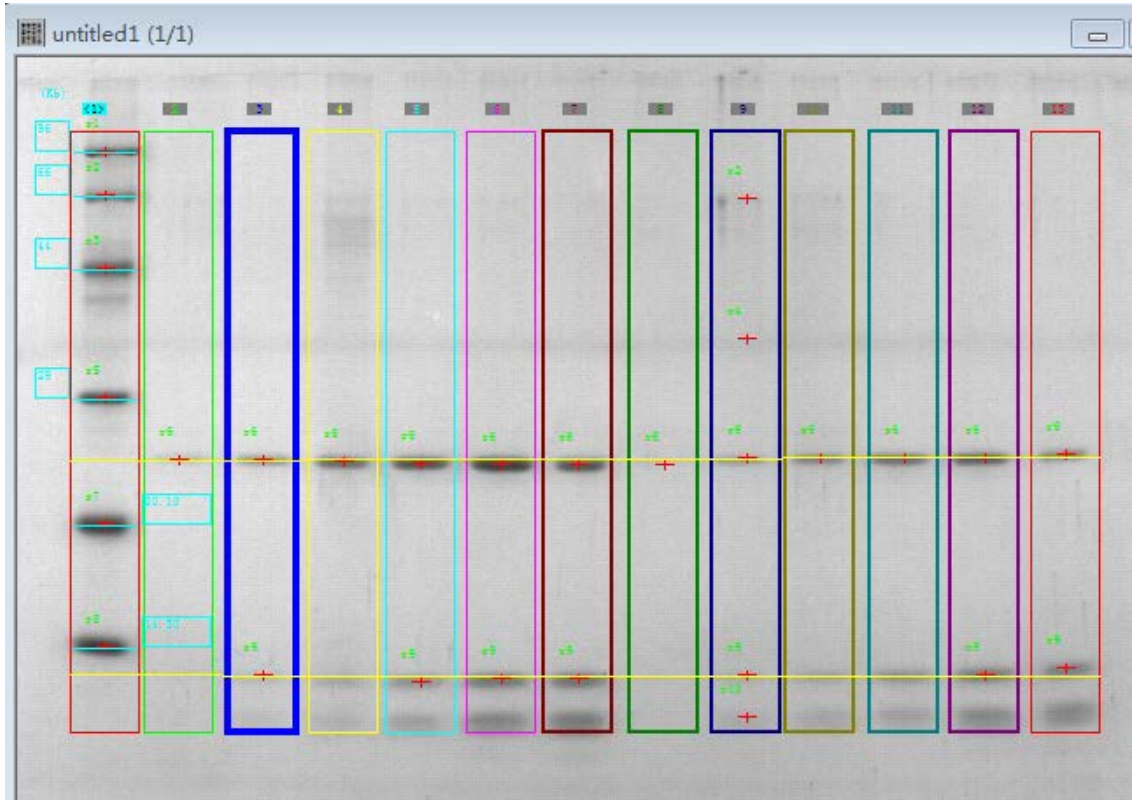
Activity of different concerntration of rTrypsin in 50mM NH_4HCO_3 pH7.8，37℃

ug/ml	10		20		50		100		200	
	U	100%	U	100%	U	100%	U	100%	U	100%
0h	110	100%	215	100%	600	100%	1140	100%	2300	100%
1h	115	105%	220	102%	575	96%	1170	103 %	2300	100%
2h	105	95%	240	112 %	540	90 %	1020	89%	2100	91 %
4h	120	109%	220	102 %	590	98 %	1160	102 %	1900	83 %
20h	75	68 %	135	63 %	290	48 %	440	39%	600	26%

Suggested used concerntration：10-50ug/ml
For example: 1ug in 50ul

Suggested digestion time: 4h

Stability of rTrypsin



1：Marker
2：10ug/ml 10h 0.2ug
3：20ug/ml 10h 0.4ug
4：50ug/ml 10h 1ug
5：100ug/ml 10h 2ug
6：200ug/ml 10h 4ug
7：500ug/ml 10h 5ug
8：10ug/ml 20h 0.2ug
9：20ug/ml 20h 0.4ug
10：50ug/ml 20h 1ug
11：100ug/ml 20h 2ug
12：200ug/ml 20h 4ug
13：500ug/ml 20h 5ug

SDS-PAGE after incubated in 50mM NH_4HCO_3 pH7.8 37℃，10h and 20h

No self-degradation.无自降解
37℃，50mM NH_4HCO_3 pH7.8 10h

Stability of rTrypsin

37℃，50mM NH_4HCO_3 pH7.8 中1mM Ca^{2+}对不同浓度胰蛋白酶的保护作用

时间h	10ug/ml		20ug/ml		50ug/ml	
	0 mM Ca^{2+}	1mM Ca^{2+}	0 mM Ca^{2+}	1mM Ca^{2+}	0 mM Ca^{2+}	1mM Ca^{2+}
0h	100%	100%	100%	100%	100%	100%
1h	100%	100%	100%	100%	100%	100%
2h	100%	100%	100%	100%	100%	100%
4h	100%	100%	100%	100%	100%	100%
20h	68%	100%	63%	79%	49%	67%

两组对照，有无1mM Ca^{2+}，37℃放置4-5h，均可保持100%的活性。

1mM Ca^{2+}对于过夜保温，有一定的保护作用，

如：20ug/ml,可避免16%的活性丧失（从63%提高到79%）；

50ug/ml,可避免18%的活性丧失（从49%提高到67%）；

10ug/ml的保护作用最明显，24h保存，可保持100%的活性，而无1mM Ca^{2+}，活性则降低了32%。

YAXINBIO Stability of rTrypsin in GuHCL

不同浓度重组胰蛋白酶在2M, 1.5M盐酸胍pH8.0, 37℃ 下的稳定性

A组：rTrypsin activity (%) in 50mM NH4HCO3 + 2M GuHCl pH8.0, 37℃

ug/ml	10	20	50	100	200
0h	100	100	100	100	100
2h	100	100	98	95	100
4h	100	100	91	1000	100
6h	100	100	89	96	86
22h	100	93	68	90	71

D组：rTrypsin activity (%) in 50mM NH4HCO3 + 1.5M GuHCl pH8.0, 37℃

ug/ml	10	20	50	100	200
0h	100	100	100	100	100
2h	100	100	100	100	100
4h	100	100	100	100	100
6h	100	100	100	100	95
22h	100	100	96	100	95

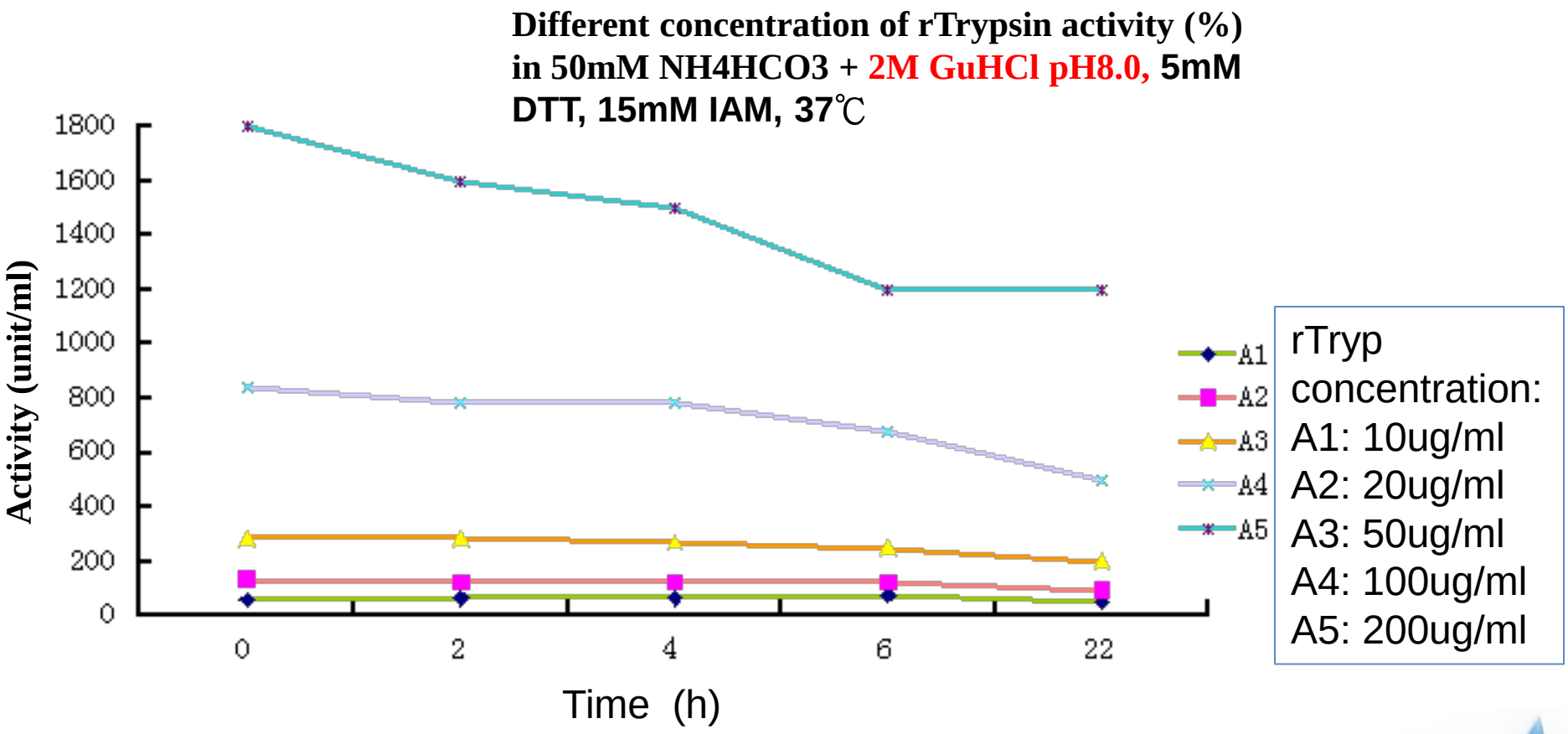
Stable in 1.5M or 2M GuHCl, pH8.0, 37℃
No self-degradation.无自降解，活性稳定

Suggested process for pretreatment and trypsin digestion:
6M GuHCl in 50mM NH4HCO3 pH8.0 for denaturing and then dilute to 2M for trypsin digestion directly.
The digestion condition:
37 ℃, 4h-5h

前处理方法：6M盐酸胍，之后稀释至1.5-2M直接加重组胰蛋白酶酶切。
酶切条件：**37 ℃, 4h-5h**

YAXINBIO Stability of rTrypsin in GuHCL

不同浓度重组胰蛋白酶在2M盐酸胍pH8.0，5mMDTTT 15mM IAM，37℃ 下的稳定性



Sequencing grade trypsin Samples：

- Promega modified trypsin 普洛麦格修饰胰蛋白酶
- YaxinBio recombinant trypsin 雅心重组胰蛋白酶

- Four aspects:

1 Cut time and Peptides recovery rate 酶切时间与肽段覆盖率

2 Missed cleavage peptides 漏切片段

3 Chymotrypsin cleavage peptides 糜蛋白酶非特异性酶切片段

4 Trypsin anto-lysis peptides 胰酶自切片段

Sample：

- **Two monoclonal antibodies**

LC-MS:

- **UPLC-Xevo G2-S QTof system (Waters)**

Mobile phase：

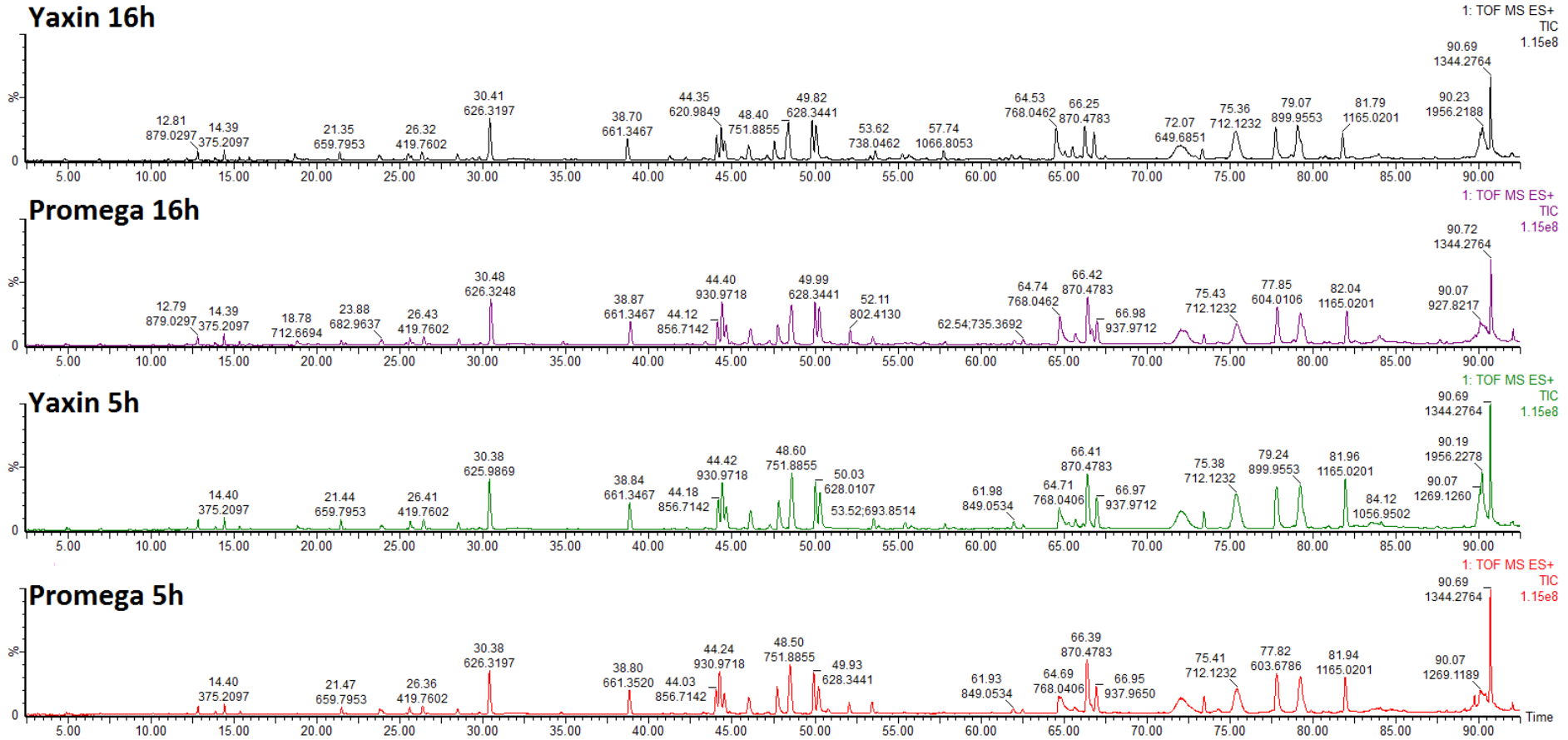
- **A, 0.1FA% water**
- **B, 0.1FA% acetonitrile**

Data processing：

- **BiopharmaLynx (Waters)**

样品前处理方法：

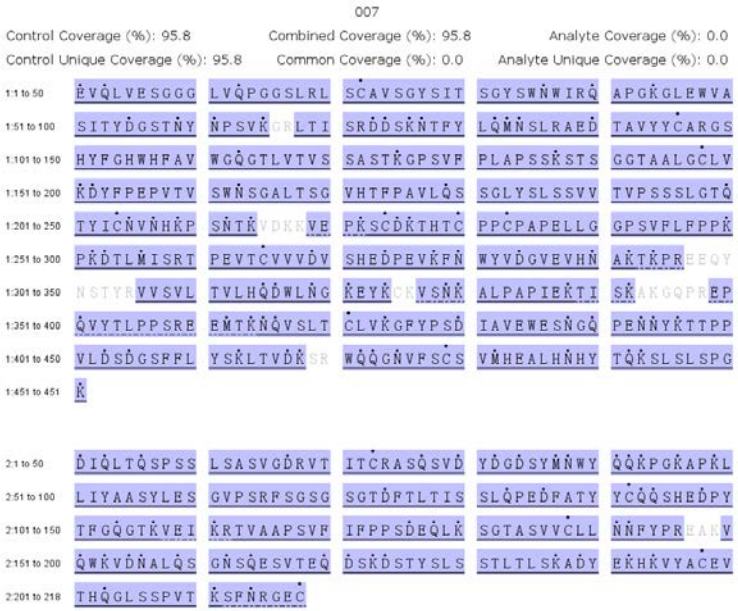
- 还原肽图样品处理（HPLC/UPLC/MAS）
- 将样品用超纯水稀释到4-5g/L，涡旋混匀，然后用6M盐酸胍，0.1mol/L Tris,Ph8.3溶液或6M盐酸胍，0.1M碳酸氢铵（AMBIC）(pH约为8.0)将样品稀释至2mg/ml, 涡旋混匀。（盐酸胍终浓度3-3.8M）
- 取稀释好的样品500ul于EP管中，加入0.5MDTT溶液5ml，涡流混匀，37℃水浴锅中水浴1.5-2.5h。（DTT终浓度5mM）
- 水浴完成后加入0.5M IAM溶液13ul，室温避光反应45min。（IAM终浓度为13mM）
- 取烷基化后的样品400ul加入到3KDa超滤离心管中，4℃，12000rpm，离心99min。
- 离心完成后，在超滤管中加入0.1M碳酸氢铵溶液150ul，4℃，12000rpm，离心60min。
- 离心完成后，将过滤部分反转于新的外管中，4℃，4000rpm，离心3min。
- 用0.1M碳酸氢铵溶液洗膜，每次用180ul，洗两次，每次都将洗液转移到外管中。
- 将上一步中的样品用移液枪轻轻吹打混匀，取100ul于EP管中，加入1ul 0.1M氯化钙溶液。（氯化钙终浓度1mM）
- 再加入胰酶溶液8ul，涡流混匀几秒钟，将样品置于37℃水浴锅中水浴酶切5、15h；（胰酶：蛋白-1:50）
- 酶解结束后，立即用1%FA样品=1:1体积比混合，终止反应。（FA终浓度0.5%）pH<5
- 10000rpm,低温离心5min，取上清液为后续分析样品。



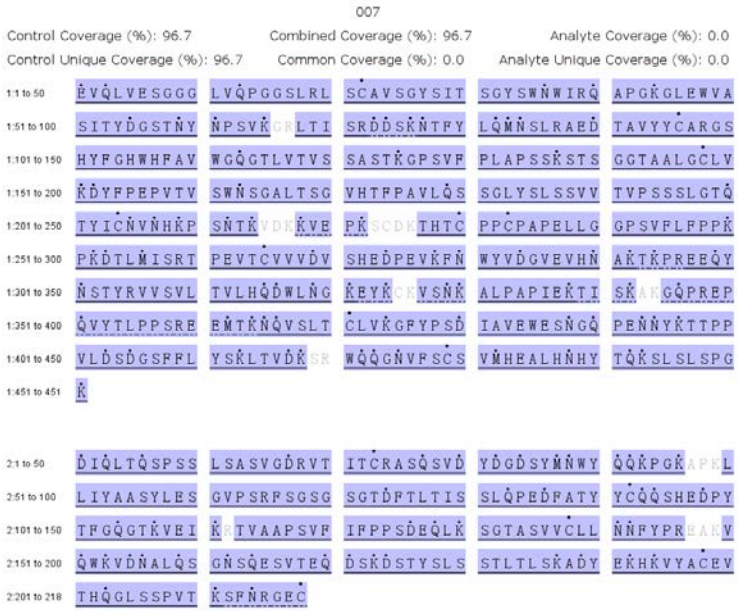
- **Almost the same chromatography profile (TIC)**

肽段覆盖率

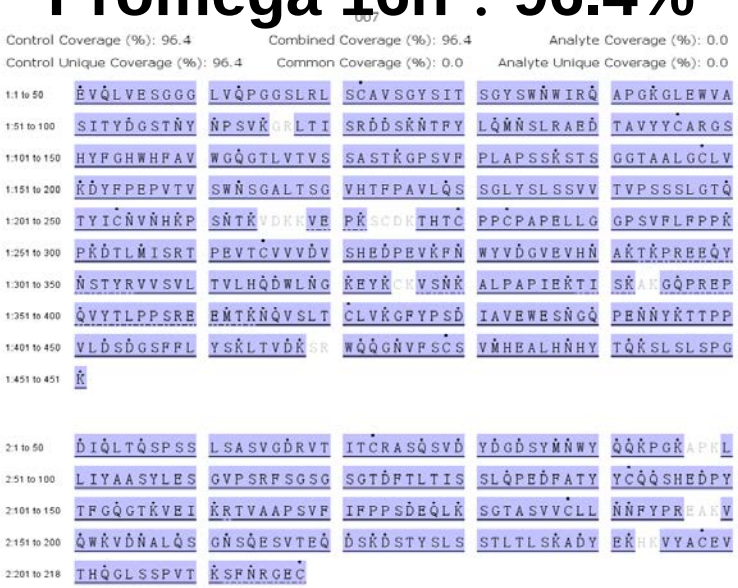
Promega 5h：95.8%



Yaxin 5h：96.7%



Promega 16h：96.4%



Yaxin 16h：97.3%

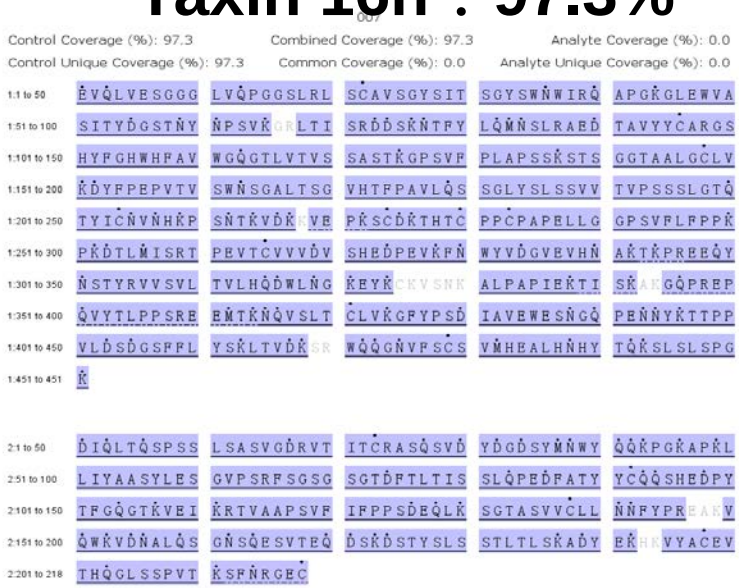
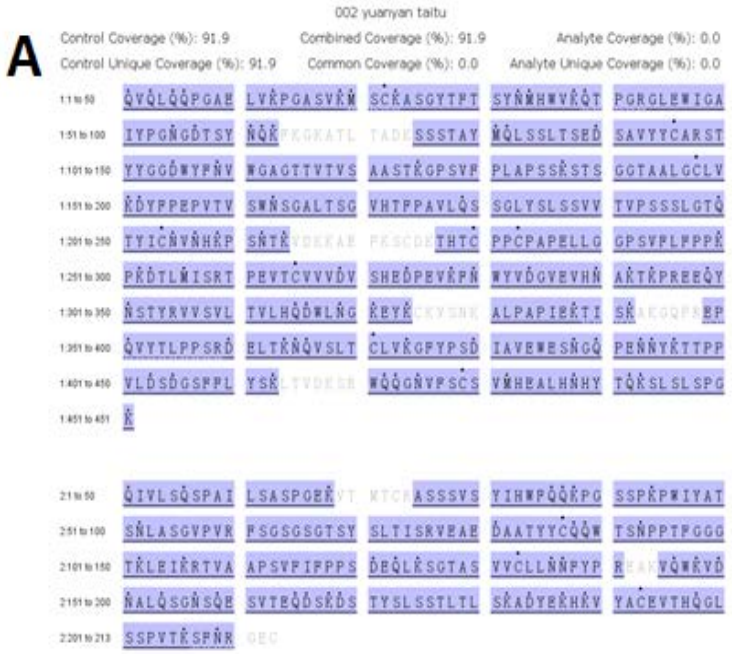


图12 002单抗胰酶酶切肽图的肽段覆盖率

A: Promega-5h 91.9%



B: Yaxin-5h 94.9%

Yaxin组胰酶酶切肽图得到更高的覆盖率。Promega实验组个别肽段碎片信息不理想而无法鉴定。Yaxin组部分肽段因为完全切开而鉴定不到，比如轻链第15-16肽段HKVYACEVTHQGLSSPVTK，HK这个肽段太短必须以漏切形式才能得到鉴定。

酶切时间与翻译后修饰鉴定

表2 主要翻译后修饰比例对比结果（007单抗）

1: T001 表示重链胰酶酶切第1个肽段；2表示轻链；

***表示因碘乙酰胺修饰半胱氨酸而产生了固定修饰**

	Promega-5h	Yaxin-5h	Promega-15h	Yaxin-15h
2:T010*	94.1%	93.6%	83.5%	83.6%
2:T010*脱酰胺	5.9%	6.4%	16.5%	16.4%
1:T039*	96.6%	96.0%	89.5%	91.7%
1:T039*脱酰胺	2.4%	3.1%	9.6%	7.4%
1:T039*脱酰胺中间体	1.0%	0.9%	0.9%	0.9%
1:T035*	96.7%	96.9%	90.7%	87.7%
1:T035*脱酰胺	/	/	9.3%	9.9%
1:T035*脱酰胺中间体	3.3%	3.1%	/	2.4%
1:T034*	98.8%	98.6%	96.1%	95.7%
1:T034*脱酰胺	1.2%	1.4%	3.9%	4.3%
1:T024	84.4%	82.7%	69.3%	69.3%
1:T024 脱酰胺	12.5%	14.6%	29.3%	29.2%
1:T024 脱酰胺中间体	3.1%	2.7%	1.4%	1.5%
1:T021	97.7%	97.2%	90.8%	90.4%
1:T021脱酰胺	1.5%	1.8%	7.7%	8.1%
1:T021脱酰胺中间体	0.9%	1.1%	1.5%	1.5%
1:T019	97.5%	97.6%	97.6%	97.6%
1:T019氧化	1.2%	1.2%	1.2%	1.3%
1:T019脱酰胺中间体	1.3%	1.2%	1.2%	1.1%
1:T008	95.3%	93.5%	83.3%	78.7%
1:T008脱酰胺	4.7%	6.5%	16.7%	21.3%
1:T001	98.2%	98.3%	98.2%	98.1%
1:T001 焦谷氨酸环化	1.8%	1.7%	1.8%	1.9%

1) LC-MS检测的另一个功能是根据质谱二级碎片结果判断并定量翻译后修饰。其中脱酰胺、氧化、赖氨酸切除等翻译后修饰是单抗质量研究中的热点。

2) 表2总结了主要的翻译后修饰所在的肽段和比例。结果显示，两种胰酶酶切肽图鉴定到的翻译后修饰种类一致，发生位点一致，相关翻译后修饰比例基本一致。

3) 值得注意的是，由于脱酰胺是一种在碱性条件下自发的化学反应，而胰酶的酶切pH一般偏碱，所以酶切时间过长会导致肽段脱酰胺的比例显著升高。

为尽量避免这种由样品前处理带来的翻译后修饰，酶切时间不宜过长。

Conclusion (I)

Cut time—4-5h

AB and cut time 单抗及酶切时间	Recovery rate	
	Promega-trypsin	Yaxin-r-trypsin
007 antibody - 5h	95.8	96.7
007 antibody -15h	96.4	97.3
002 antibody - 5h	91.9 (should less due to one identified as missed peptide)	94.9 (should high due to one HK too short to be identified)

另外，由于脱酰胺是一种在碱性条件下自发的化学反应，而胰酶的酶切pH一般偏碱，所以酶切时间过长会导致肽段脱酰胺的比例显著升高。

为尽量避免这种由样品前处理带来的翻译后修饰，酶切时间不宜过长。

Promega-Tryp & R-Tryp Comparation (2)
---Missed cleavage peptides 漏切片段

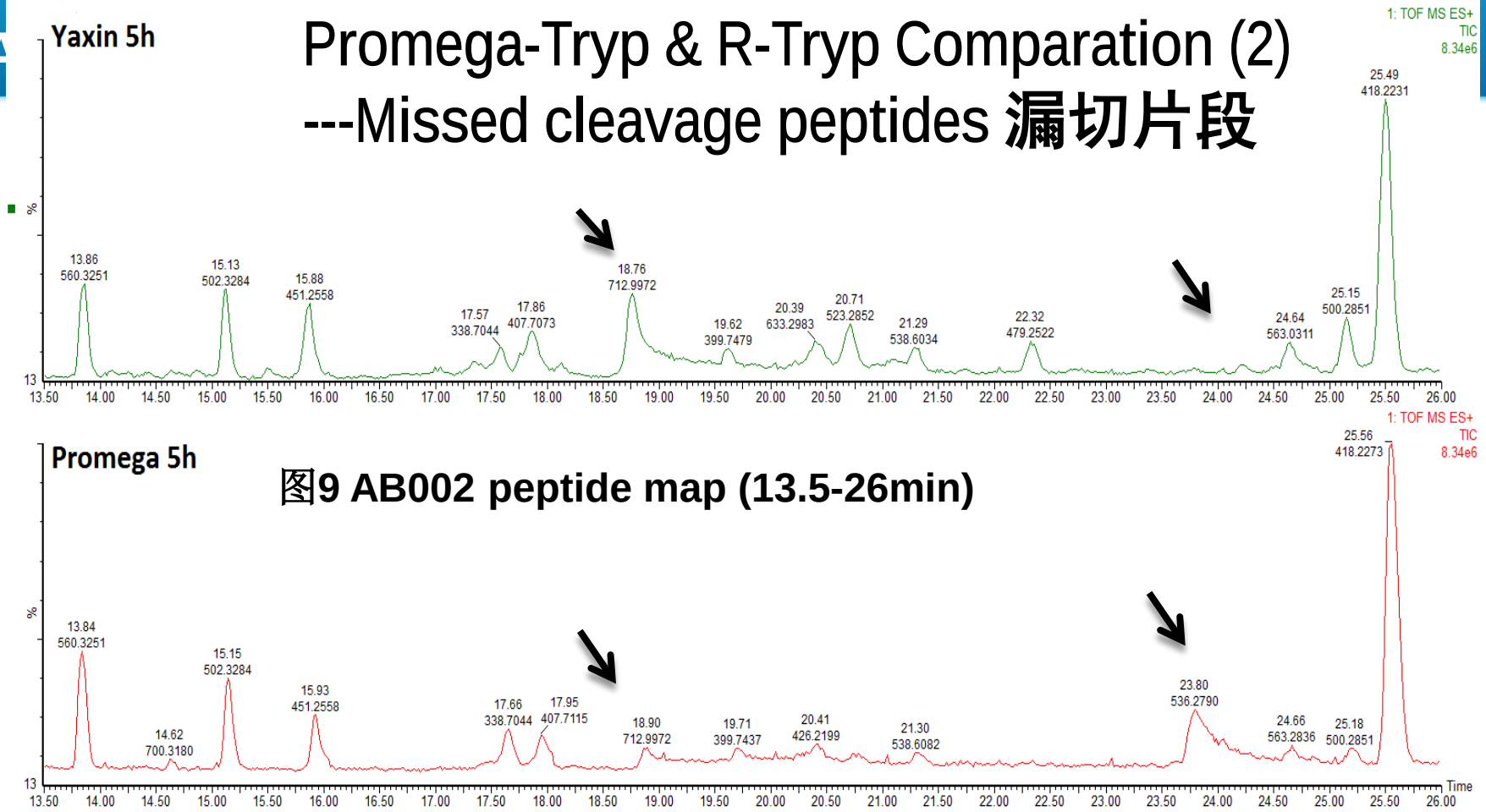


图9 AB002 peptide map (13.5-26min)

（1）漏切片段太长：**18.9min**峰是轻链正常酶切的第**12**肽段。**Yaxin**组酶切获得更多的轻链第**12**肽段，而**Promega**组可能是存在漏切导致部分轻链第**12**肽段仍然连在上一段或下一段肽段上，这两个漏切肽段都太长而检测不到。

（2）以漏切片段签定：**23.80min**峰是轻链漏切一个位点的第**15-16**肽段。**HKVYACEVTHQGLSSPVTK**，**HK**这个肽段太短必须以漏切形式才能得到鉴定。对于第**15-16**肽段，**Yaxin**组完全切开，而**Promega**组存在一定量的漏切肽段。

图10软件处理后的002单抗胰酶酶切肽图图谱（Promega 5h）

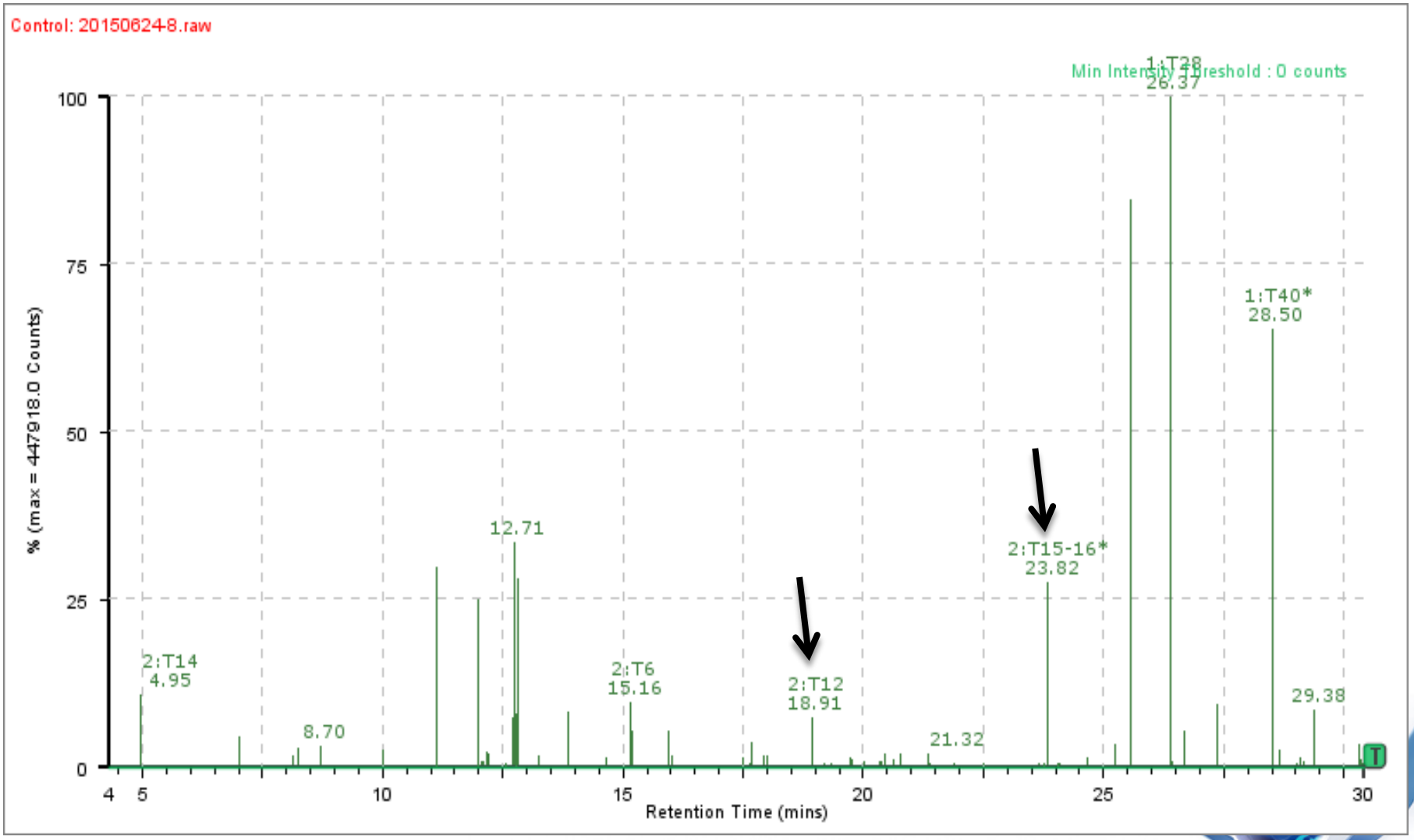
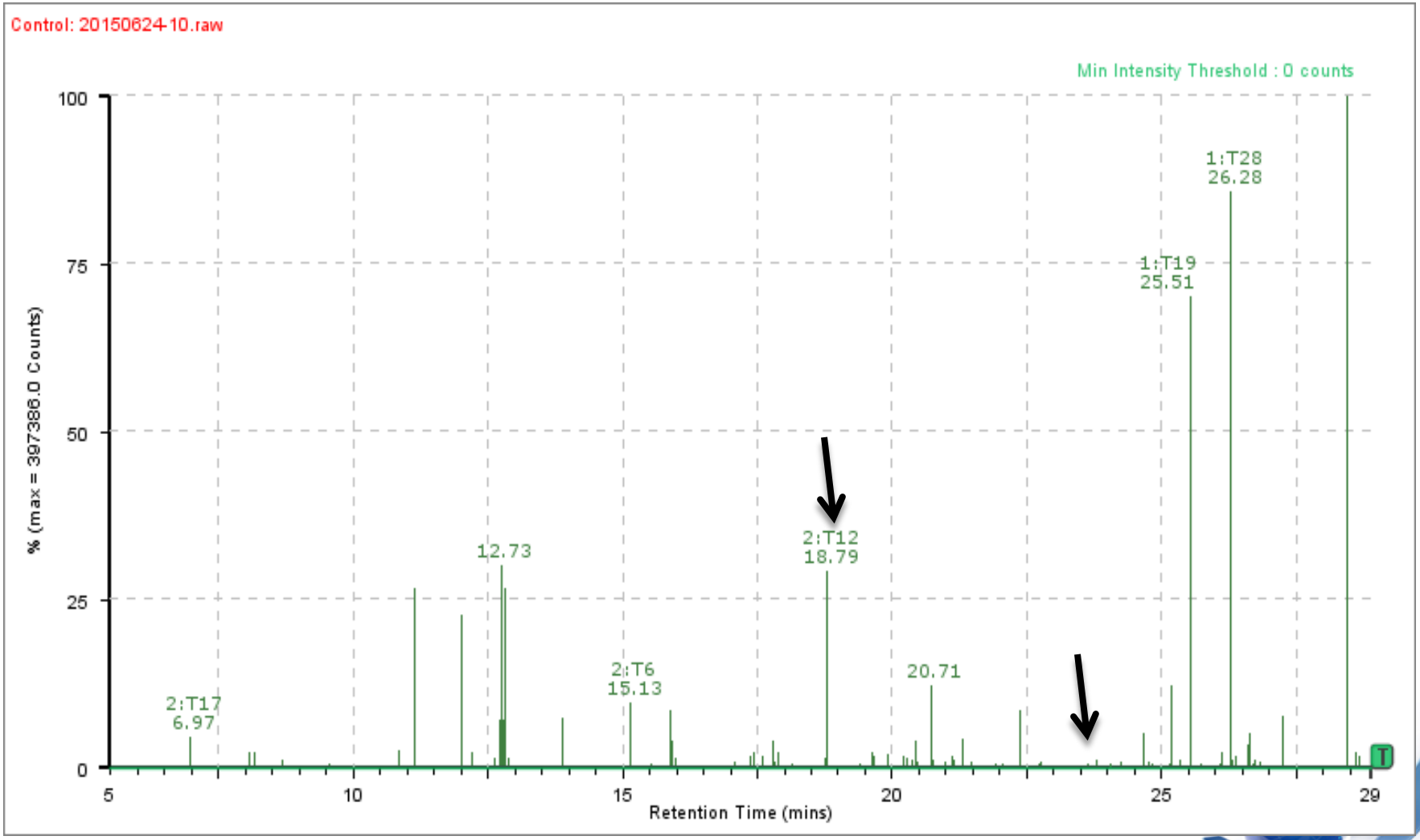


图11软件处理后的002单抗胰酶酶切肽图图谱（Yaxin 5h）



Promega-Tryp & R-Tryp Comparation (2)
---Missed cleavage peptides 漏切片段
Conclusion (2)

(1)Higher recoverage rate is got with Yaxin R-trypsin 94.9% to 91.9%
Actually, higher than 94.9% due to P15-16 HK is too short to be identified.
(2) Less missed cleavage peptides
Such as P12, P16

(1) 对于第15-16肽段，Yaxin组完全切开，而Promega组存在一定量的漏切肽段。因此在相同酶切条件下，Yaxin胰酶的酶切效率更高且5h足以酶切完全。
(2) 两组肽图覆盖率为91.9%和94.9%。Yaxin组胰酶酶切肽图得到更高的覆盖率。Promega实验组个别肽段碎片信息不理想而无法鉴定。Yaxin组部分肽段因为完全切开而鉴定不到，比如轻链第15-16肽段HKVYACEVTHQGLSSPVTK，HK这个肽段太短必须以漏切形式才能得到鉴定。

Promega-Tryp & R-Tryp Comparation (3)

---Chymotrypsin cleavage 糜蛋白酶酶切

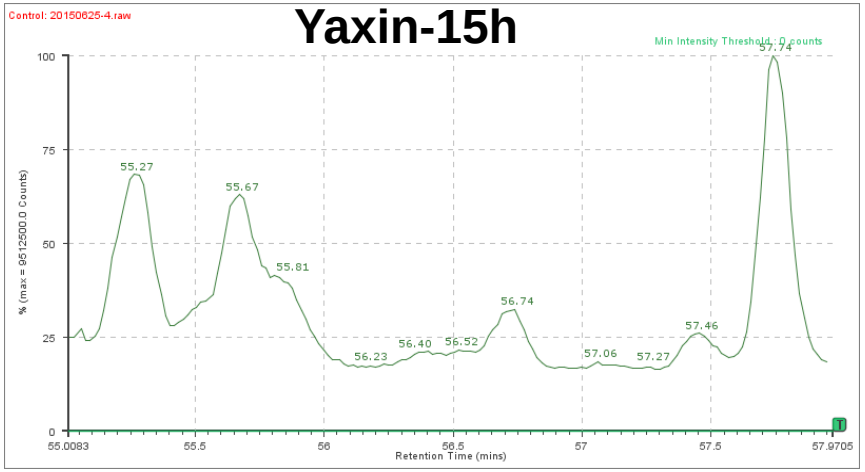
Table 1 chymotrypsin cleavage peptides

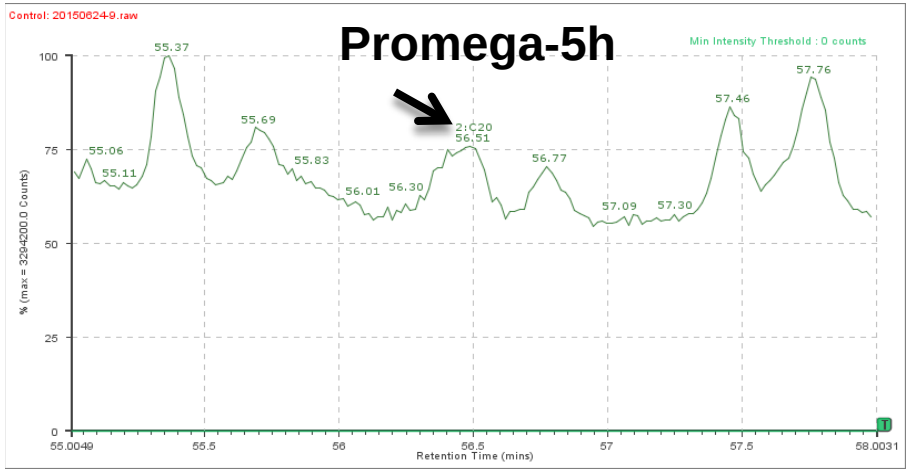
007 AB/signal strength	Promega-5h	Yaxin-5h	Promega-15h	Yaxin-15h
Heavy Chain C020	19002	None	90655	None
Heavy Chain C002	4407	None	23853	None
002AB/signal strength				
Heavy Chain C020	29613	None	/	/

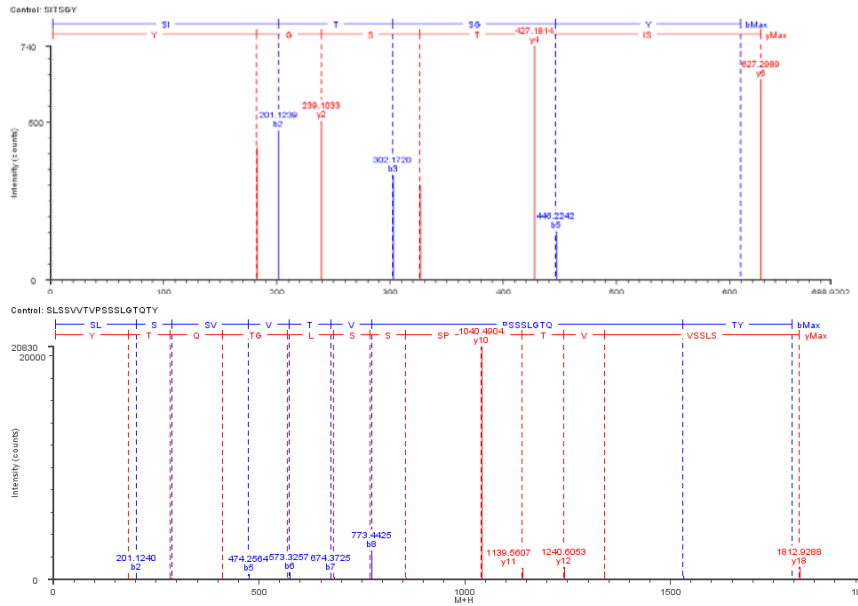
C020: the 20th peptide fragment of the aimed antibody by chymotrypsin
表示糜蛋白酶酶切抗体蛋白产生的第二十个肽段

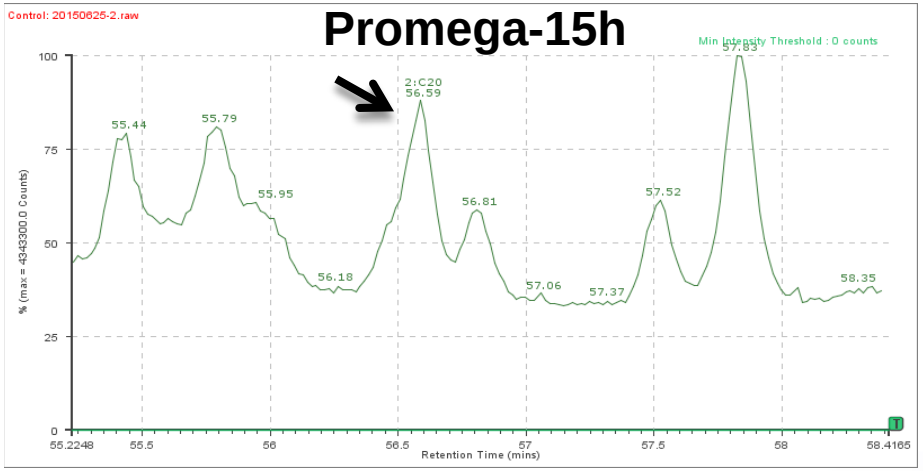
通过软件检索，发现Promega胰酶处理的单抗肽图中存在糜蛋白酶的特异酶切肽段。且信号强度随着反应时间的延长而增强。007单抗的重链C002，出峰时间18.2min，序列为SITSGY属可变区，因而在002单抗肽图中没有找到。重链C020，出峰时间56.6min，序列为SLSSVVTVPSSSLGTQTY属恒定区，在两个单抗的肽图中都能发现。

Promega-Tryp与R-Tryp酶切效果比较3









007单抗重链C020肽段局部区域图谱

007单抗重链C020肽段二级碎片图谱

Promega-Tryp & R-Tryp Comparation (3)

--- trypsin auto-cleavage 胰蛋白酶自切

表2胰酶自切肽段（007单抗）

肽段/信号强度	Promega-5h	Yaxin-5h	Promega-15h	Yaxin-15h
T007	30970	66064	46149	106509
T006	5887	41032	8934	71102
T005	9011	234266	8014	244695
T004	23456	180728	30468	435894

T007表示根据胰酶理论肽谱图排序胰酶自切产生的第七个肽段

1) 均有4个相同的自切片段。
2) 但可以明显看到雅心重组胰酶的自切现象比Promega动物提取胰酶严重，尤其是T005和T004肽段含量高出很多，

TPCK-Tryp与R-Tryp酶切效果总结

总结：

1）两种单克隆抗体酶切肽图结果表明，Yaxin胰酶可达到Promega胰酶的效果；

2）两种胰酶酶切5h都可获得理想的肽段覆盖率；

3）酶切5h，Yaxin胰酶的酶切效率比Promega胰酶高；

4）若想获得100%肽段覆盖率，应选择另一种蛋白酶的酶切肽图作为补充。

盐酸胍变性后稀释酶切

前处理方法：

样品经盐酸胍变性、还原烷基化处理后，用pH=8的碳酸氢铵溶液将蛋白溶液稀释至盐酸胍为1M和2M。随后以1:50（酶：重组蛋白）的比例加入胰酶，置于37℃水浴锅反应。酶切5h和16h分别检测。所有对比试验条件包括蛋白浓度，酶切反应温度，进样量等等均保持一致，只有胰酶不同。

盐酸胍变性后稀释酶切

条件		Promega Modified Trypsin	YaxinBio-rTrypsin
1M盐酸胍	5h	90%	91.6%
	15h	91.6%	88.2%
2M盐酸胍	5h	79.8%	92.4%
	15h	90%	88.8%

1M 盐酸胍，酶切5h都能得到理想的覆盖率，Promega组没有找到重链第32肽段EPQVYTLPPSR，而Yaxin组能找到。延长酶切时间至15h，Promega组也能找到该肽段。说明Promega胰酶需要更长的时间才能切出这个肽段。这与超滤无尿素酶切的实验结果一致。

2M 盐酸胍，Yaxin组观察到，覆盖率随着酶切时间延长而减少。这是因为诸如重链第17-18肽段SCDKTHTCPPCPAPELLGGPSVFLFPPKPK，轻链第18-19肽段SFNRGEC随着酶切时间延长而完全切开，产生重链第17肽段SCDK、轻链第18肽段SFNR、轻链第19肽段GEC。这种短肽在色谱柱上往往保留很差而鉴定不到，造成覆盖率减少。Promega组延长酶切时间，这些肽段仍以漏切形式得到鉴定，表明该酶无法将漏切肽段完全切开。

盐酸胍变性后稀释酶切

1）对比超滤除盐实验组的肽图覆盖率可知，在含盐酸胍情况下胰酶酶切肽图鉴定的覆盖率（90%-91%）低于超滤除盐实验组（95%-96%）。分析两者差异，主要是一些漏切肽段和糖基化肽段EEQYNSTYR在含盐酸胍实验组中没有被鉴定到。漏切肽段一般情况下量比较少，

2）对比发现尽管蛋白进样量一致，含盐酸胍实验组的质谱信号几乎低了1倍。这说明可能是盐酸胍也可能是单抗样品原液中的某些辅料或杂质抑制了总体质谱信号，导致含量本就较低的一些漏切肽段检测不到。而糖基化肽段的鉴定方法一般是先用糖苷酶切除糖链，使其转变成普通肽段才能得到鉴定

盐酸胍变性后稀释酶切对比结果

总结：

1） 在含有盐酸胍的溶液中，Yaxin重组胰酶的酶切效率高于Promega提取胰酶；

2）在1M盐酸胍浓度下，两种胰酶酶切5h均能得到较理想的肽图覆盖率。在2M盐酸胍浓度下，Yaxin胰酶酶切5h即可得到较理想的肽图覆盖率，而Promega胰酶需要更长的酶切时间；

3） 进样同样的蛋白含量，含盐酸胍实验组的质谱信号低于超滤除盐的实验组；

4）由于质谱信号较低，含盐酸胍实验组的诸多比例较低的翻译后修饰肽段未能检测到。

C-Lysine certificate of AB

Enzyme: recombinant Carboxypeptidase B

有关CPB碱性峰

CpB碱性峰意思就是说，如果原研药酸峰30，主峰50，碱性峰20。而自己样品的酸峰30，主峰45，碱性峰25。cpb处理后，原研药酸、主、碱峰仍是30，50，20。自己的样品也是30，50，20了。那么说明，自己样品与原研药只差5%的赖氨酸变体导致的碱性峰。这个在申报上说清楚就可以了，国外做过实验的这对安全性有效性没有影响的。欧盟有这个案例的仿制药，也都批准上市了。所以CpB在抗体电荷检测应用上是一定要用的。

酸性峰 certificate of AB

Enzyme: V8

天冬氨酸酶，谷氨酸酶

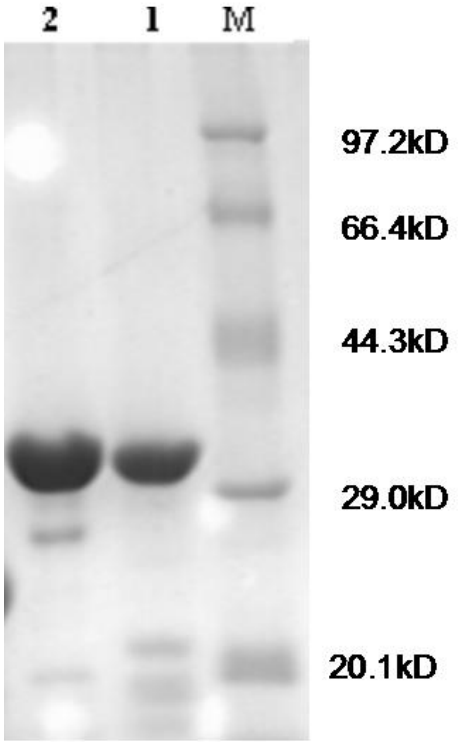
抗体检测也需要鉴定酸性峰，以对照脱酰胺程度。抗体最重要的是Asn的的脱酰胺形成Asp。

用到V8酶。

V8特异性酶切Asp和Glu的C端所形成的肽键。

YAXINBIO C-Lysine certificate of AB

Enzyme: recombinant Carboxypeptidase B



1, Merck CPB
2, YaxinBio rCPB

ITEMS	SPECIFICATION
Source	Recombinant *E.Coli*
Purity	> 95%
Specific activity	>200 U/mg pro.
Trypsin content	<1ppm
Other enzymes	None
Form	Lyophilized powder
pI	5.4

STORAGE AND STABILITY

Recommend recombinant carboxypeptidase B lyophilized powder stored under 2-8℃ in sealed container. It is stable within 24 months.

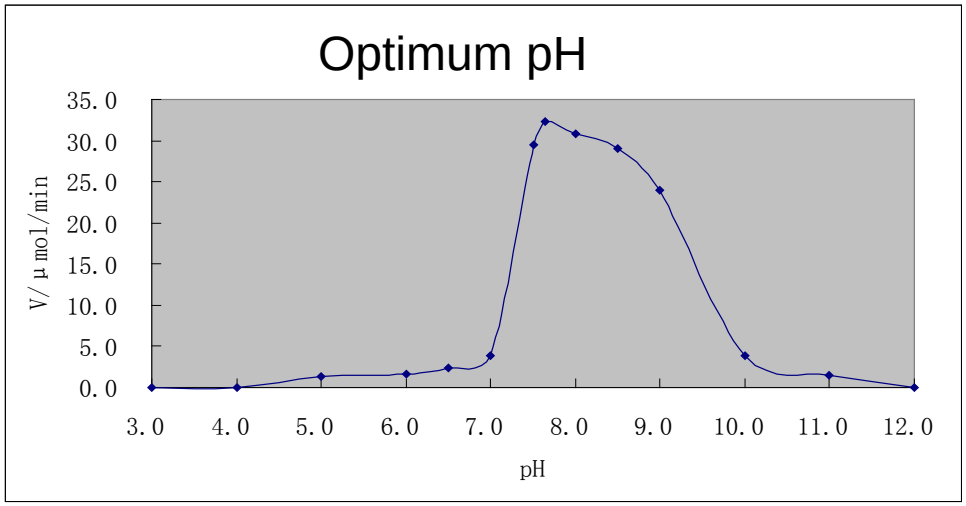
After dissolved, it should be stored under -20℃, It is stable within 24 months and no activity lose after 10 times repeated freezing and thawing.

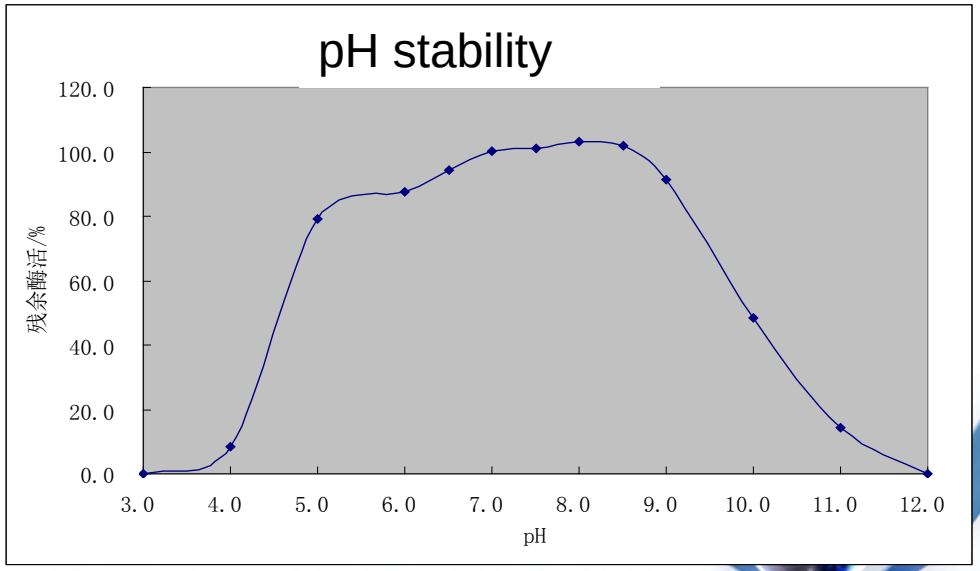
C-Lysine certificate of AB

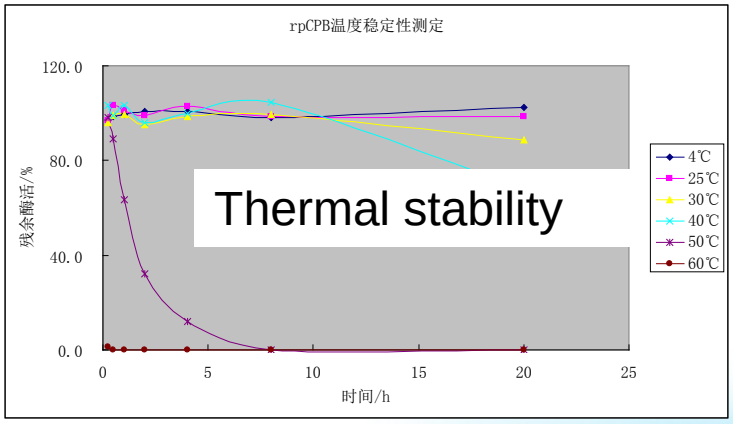
Enzyme: recombinant Carboxypeptidase B 重组羧肽酶B

Suggested methods：
Enzymatic pH: 7.5-9.0
Optimum pH: 7.6
Analysis HPLC pH: 6.0-9.0
Time: 1h--3h
Temperature: 25℃-37 ℃

建议使用方法：
酶解时pH：7.5-9.0,
最适pH:pH7.6
分析时pH：6.0-9.0
酶解时间：1h--3h
酶解温度：25℃-37 ℃

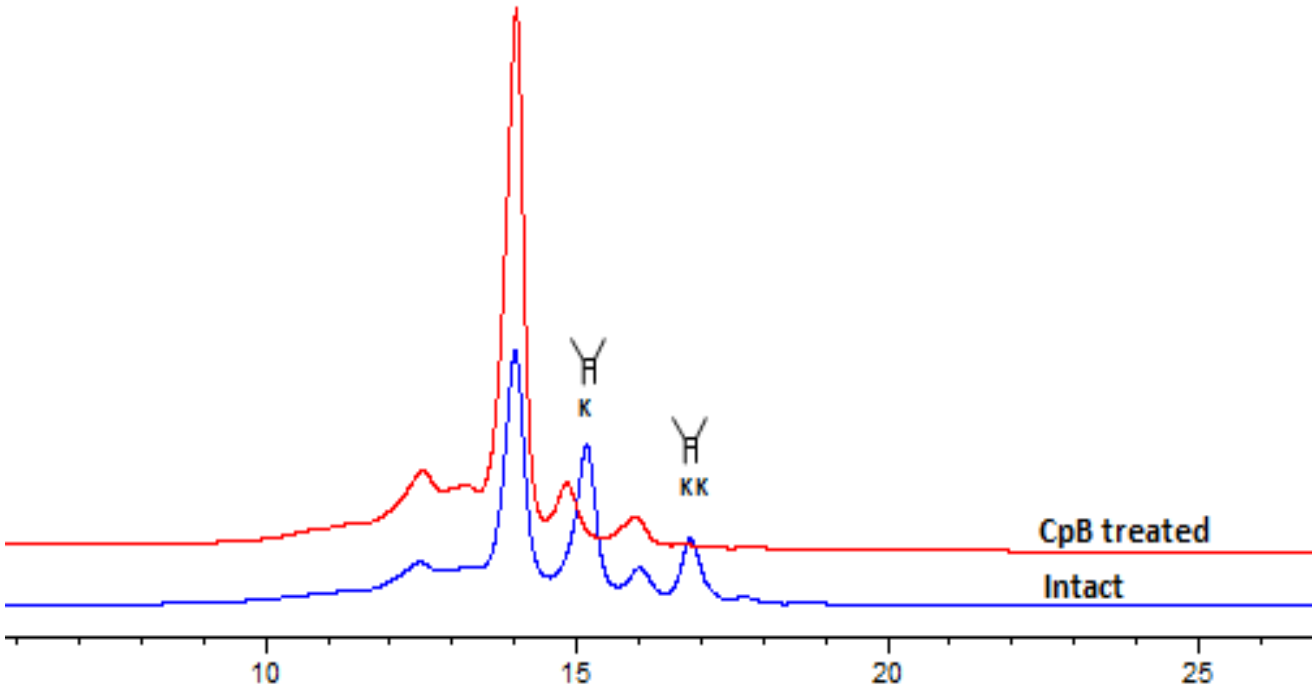






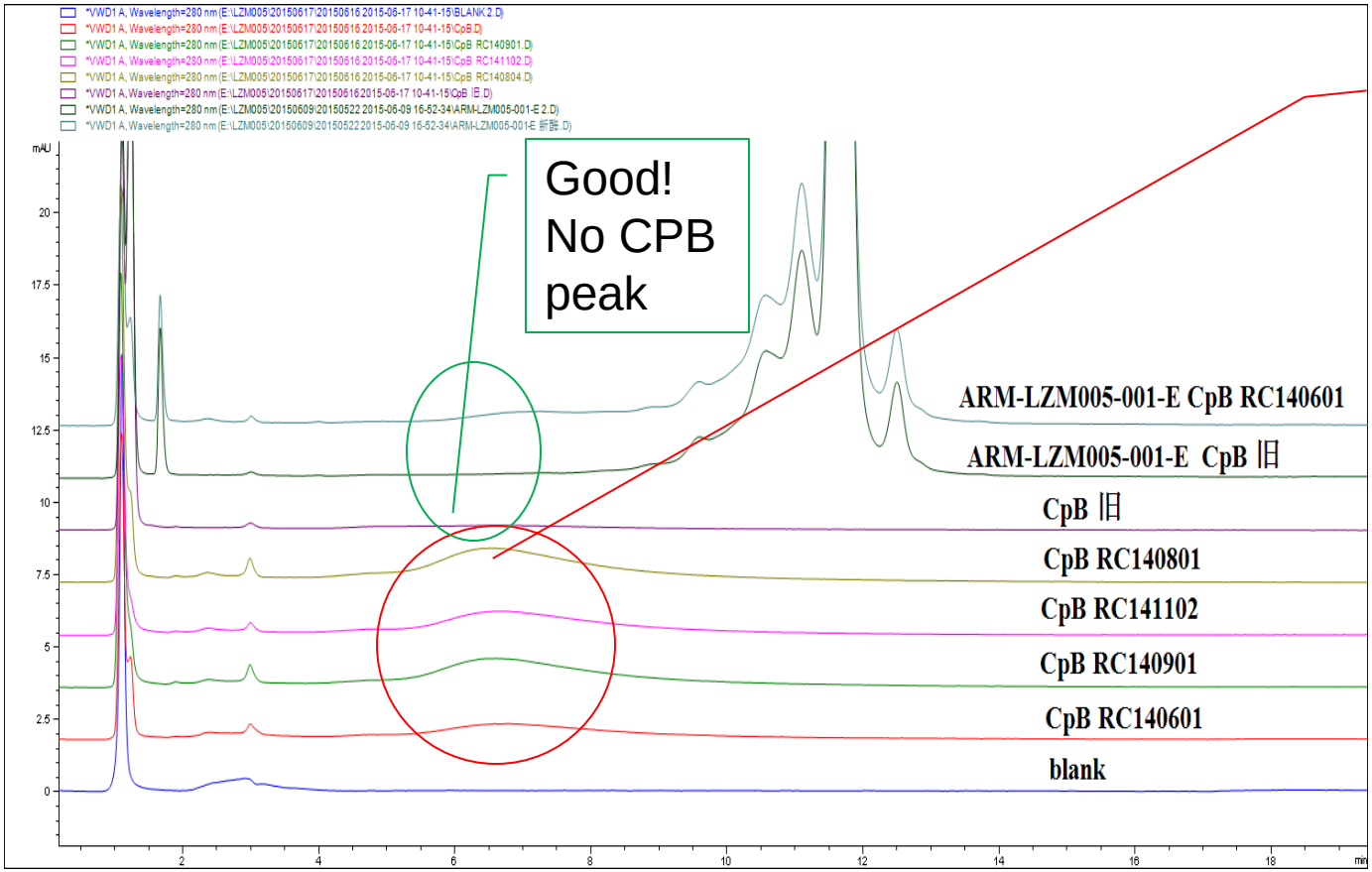
举例：

羧肽酶B（RCPB）：Yaxinbio RC01。
用1XPBS溶解羧肽酶B至1g/L，分装后-20℃保存。
纯化后的单抗样品超滤至磷酸缓冲液（pH=7.5）中（1~2g/L），
按样品：酶=100:1比例加入羧肽酶B，37℃反应30分钟后直接进样HPLC检测。



YAXINBIO C-Lysine certificate of AB

Enzyme: Carboxypeptidase B 用量不要太大。



CPB peak due to much more CPB used (2.6ug in loading sample).

Suggested methods：
Ratio of AB to CPB： 20:1 to 100:1
Used concentration： 0.5ug-1ug in 50ul

YAXINBIO 测序级糜蛋白酶 r-chymotrypsin

Source	*E. Coli*
Purity	NLT 95% by HPLC analysis.
Physical form	Lyophilized powder
Specific activity	NLT 1500 USP units/mg pro.
No Contaminant activity	No carboxypeptidase A, or other proteases contaminant.

APPLICATION

Protein digests for peptide mapping applications or protein identification by peptide mass fingerprinting or MS/MS spectral matching. It is suitable for digestion reactions in-solution or in-gel.

RECOMMEND USAGE

To prepare 1-10mg/ml with 1mM HCl, used within 2 days, or stored below -20℃ after repacked

STORAGE AND STABILITY

Recommend recombinant Chymotrypsin lyophilized powder stored under 2-8℃ in sealed container. It is stable within 24 months. After dissolved, it should be stored under -20℃.

Main Products

Sequencing grade enzymes for protein analysis

Cat. No.	Product Name	Function	Charaters	Package
SRCPB0102	Recombinant Carboxypeptidase B (Sequencing grade)	---↓Lys/Arg/His	Specific Activity (unit/mg)	100μg
			NLT 200	1mg
SRT0202	Recombinant Trypsin (Sequencing grade)	---Lys/Arg↓---	Specific Activity (BAEE unit/mg)	100μg
			NLT 15000 BAEE unit	1mg
SRCT10	Recombinant Chymotrypsin (Sequencing grade)	---Tyr/Phe/Trp↓--- at a lower rate, at Leu and Met.	Specific Activity (unit/mg)	100μg
			NLT 1500	1mg
V813	V8 (Endoproteinase Glu-C) (Sequencing grade)	---Glu/Asp↓---	Activity (unit/mg)	50μg (1U)
			20	2mg

YAXINBIO Animal free questions in vaccine

The first is : Non-serum media

The second is : recombinant trypsin

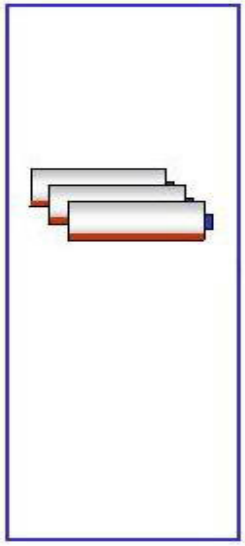
rTrypsin is to meet the needs of the cell culture and vaccine industry. With same performance to animal derived trypsin, rTrypsin provides efficient dissociation of cells from surfaces and tissues while maintaining cell viability and integrity.

Additional question is : recombinant trypsin inhibitor

Vaccine production 疫苗生产

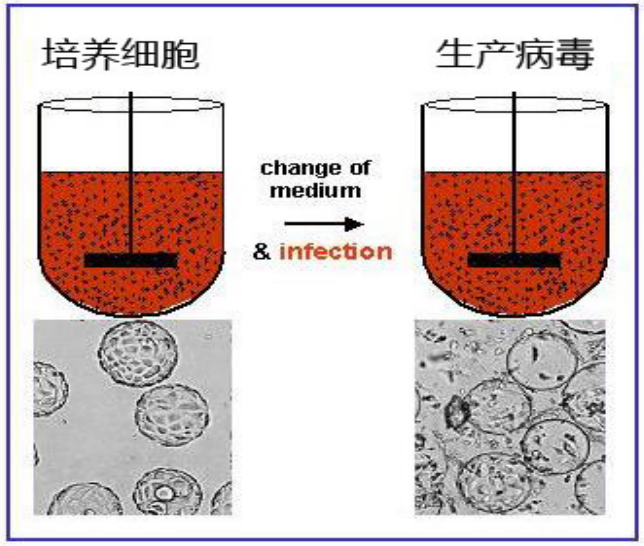
Turn flasks culture

滚瓶培养

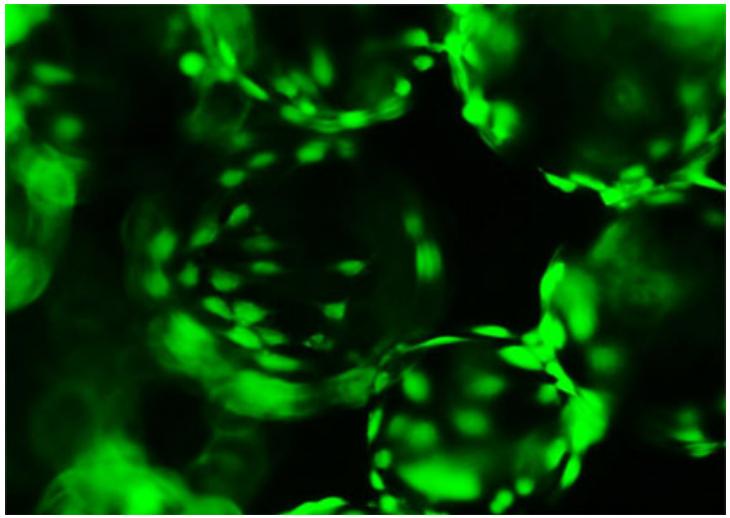


Micro carries culture

微载体培养



Micro carries culture



接种—初期培养—贴壁阶段—收获细胞—扩培

Vaccination, early training, stick a wall stage - harvest cell - culture

Trypsin is used during harvesting,
If propagate after harvesting, aprotinin is needed to inhibit trypsin activity.

YAXINBIO Trypsin history used in the vaccine production

◆ Trypsin (EC3.4.21.4) has been available for many years as native enzyme isolated from porcine and bovine pancreas.

➢ 1:250 trypsin specially for cell dissociation

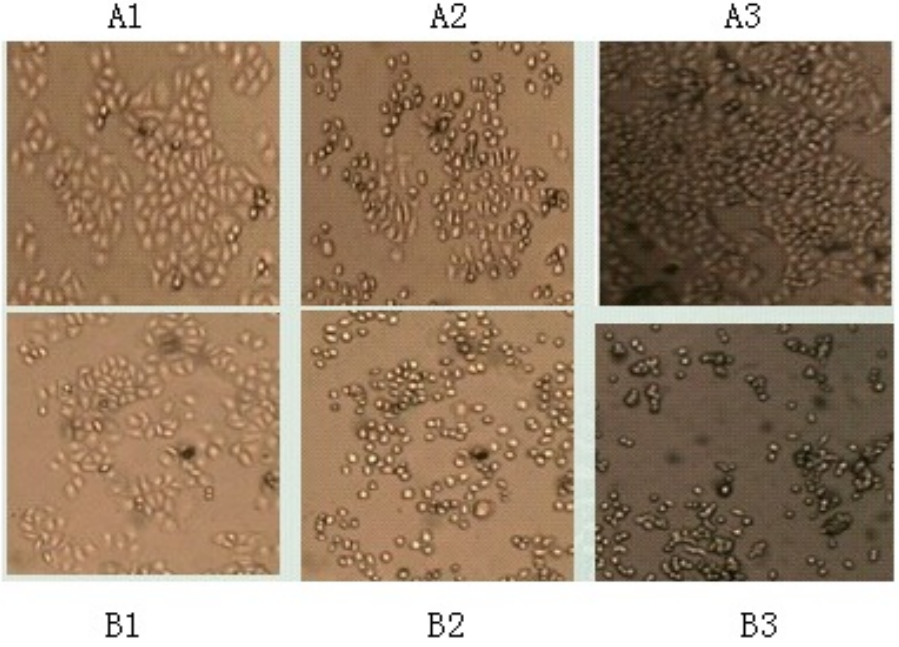
➢ 1:2500 trypsin collected by CP, USP, EP and JP etc in 1970s.

➢ 1:3800 recombinant trypsin issued by USP in 2014

➢ In 2015, CP2015 is preparing the standard for recombinant Trypsin.

RT used in cell culturing

Hela Cell **YaxinBio R-Trypsin**



A: rTryp1000 BAEE units/ml+0.01% EDTA

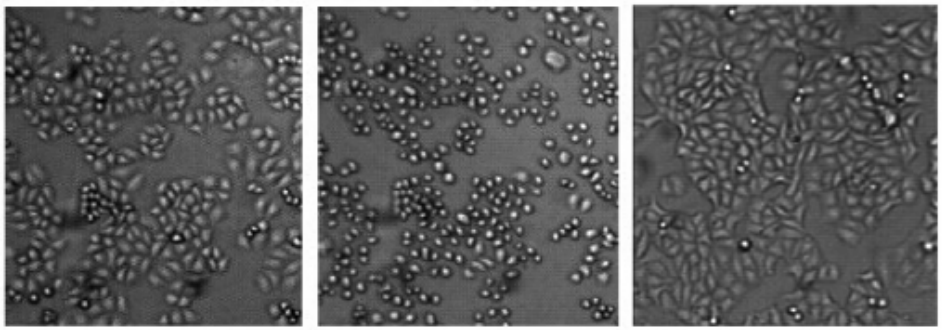
A1：before；A2：after 3min with rT；

A3：after 15h culturing

B: rTryp10000 BAEE units/ml+0.01% EDTA

B1：before；B2：after 30sec with rT；

B3：after 15h culturing



C: rTryp2000 BAEE units/ml+0.01% EDTA

C1：before；C2：after 2min with rT；

C3：after 24h culturing

RT used in cell culturing

The effects of Recombinant trypsin components for cell detachment and its growth

Components	Time (min) for detachment	48 h Cell Growth condition
1000 BAEE units in PBS	7	+++
2000 BAEE units in PBS	6	+++
5000 BAEE units in PBS	4.5	+
10000 BAEE units in PBS	3	-
1000 BAEE units in PBS + 0.01% EDTA	3	+++++
2000 BAEE units in PBS + 0.01% EDTA	2	+++++
5000 BAEE units in PBS + 0.01% EDTA	1.8	+
10000 BAEE units in PBS + 0.01% EDTA	1.5	-

Recombinant Trypsin: YaxinBio
Cell Line: HeLa

Keys for trypsin usage

1. The concentration of trypsin

If shift from 1:250 trypsin, convert with activity, such as,

if 0.25% for 1:250 (250 USP unit/mg),

to 0.025 for 1:2500 (2500 USP unit/mg),

to 0.016 for 1:3800 (3800 USP unit/mg).

2. The time for trypsin

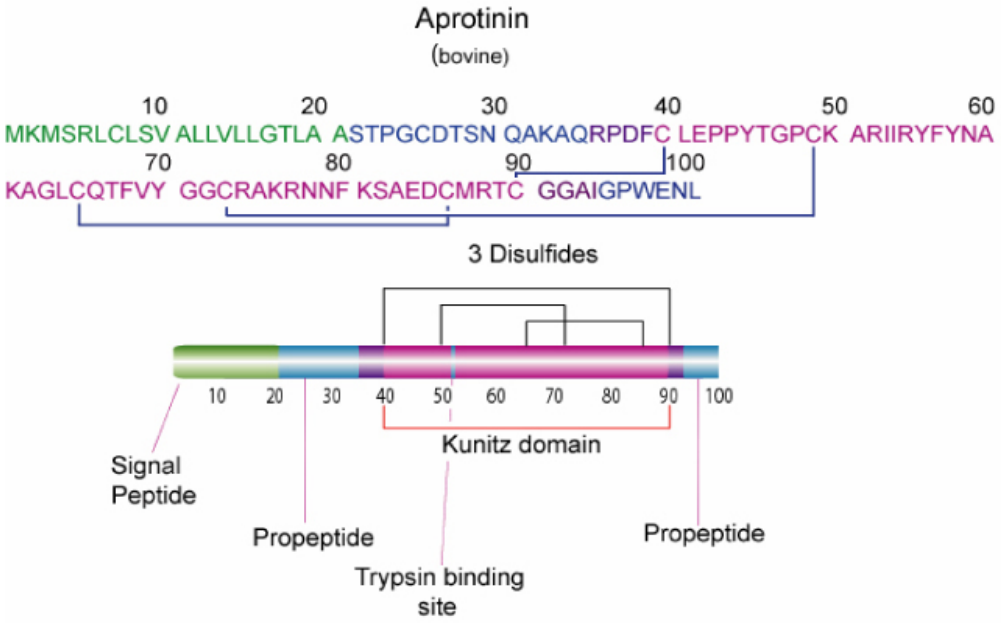
If change FBS to non-serum medium, pay attention to the time, to stop the trypsin with inhibitor.

3. Different cell line owns different optimum concentration of trypsin, find a optimum low effective concentration

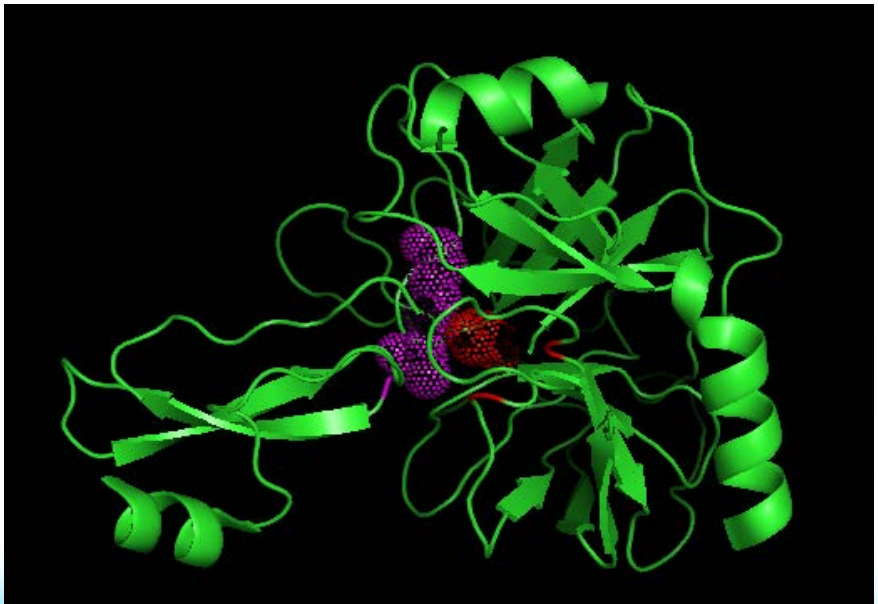
Trypsin Inhibitor

➢ Aprotinin /BPTI

While aprotinin and bovine pancreatic trypsin inhibitor (BPTI) are the same protein sequence, the term aprotinin is typically used when describing the protein derived from bovine lung.



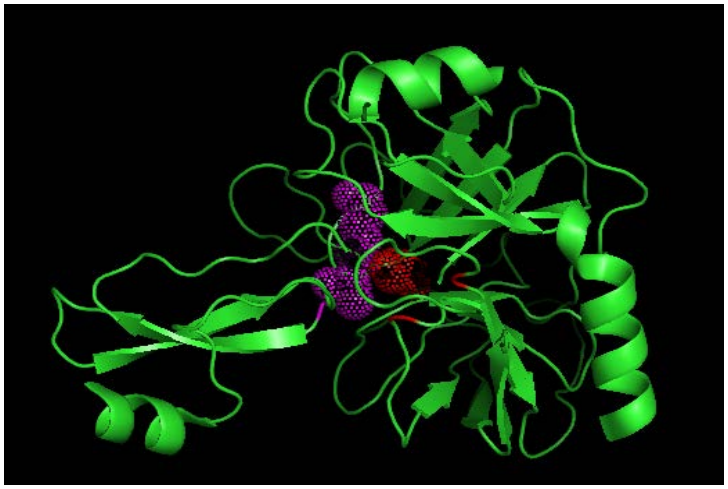
58 amino acid, 3 disulfides



Trypsin Inhibitor

- recombinant Aprotinin /BPTI --YaxinBio

Source	*E. Coli*
Purified by	HPLC
Physical form	Liquid in 0.1 M NaCl or White lyophilized powder
Specific activity	≥ 5 EPU/mg pro.
Purity	≥ 98% by SDS-PAGE
Contaminant activity	No any other protease contaminant.



Aprotinin-trypsin complex: mol:mol

UNIT DEFINITION

One trypsin inhibitor unit (EPU) will decrease the activity of 2 trypsin units by 50% where one trypsin unit will hydrolyze 1.0 µmole of N-benzoyl-L-arginineethyl ether (BAEE) per sec at pH 7.6 at 25 ℃.

A conversion factor for Aprotinin is: 1 EPU = 1 USP Aprotinin Unit = 1800 KIU.

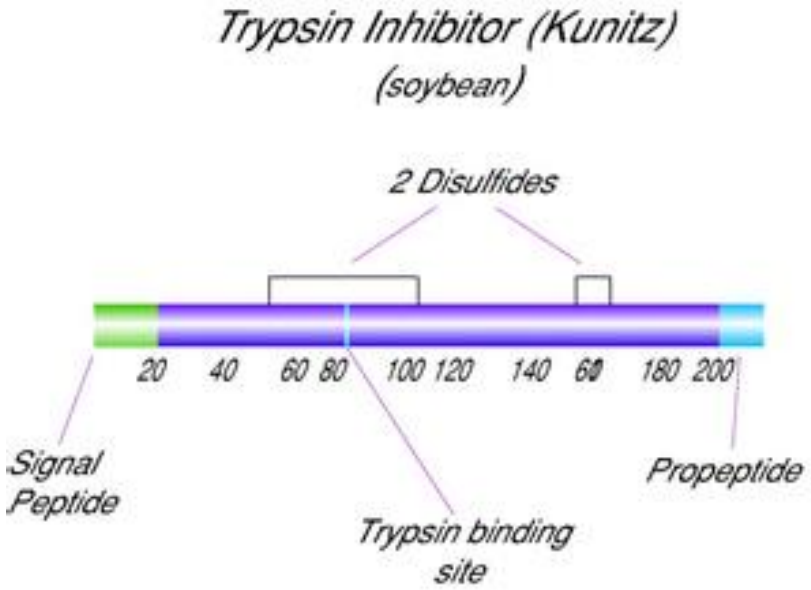
Usage: equal- mol trypsin (or 1/3 weight of trypsin)

store condition：2-8℃ or –20 ℃

Trypsin Inhibitor

- soybean trypsin inhibitor
  ---from TrypZean sigma

Soybean trypsin inhibitor and other inhibitors work the same with TrypZean as they do with native trypsins (on a weight-to-weight basis)



Activity: >7,000 BAEE units/mg
Usage suggestion: 0.25-0.5 mg/ml in PBS.
I**nhibit** equal- volume trypsin
store condition：–5 ℃ -- –20 ℃

180 amino acid, 2 disulfides

Enterprise Vision

- **Manufacturer of Animal Components Free recombinant proteins with *Eschericoli* system.**

  致力于生物医药用、无动物源性的重组蛋白和重组蛋白酶的研发和生产

- **Supplier of recombinant protein to Biopharmaceutical company.**

  为国际生物制药公司提供重组蛋白和重组酶。

- **Optimal individualized**

  为生物制药客户提供最优的个性化服务。

SHANGHAI YAXIN BIOTECHNOLOGY LIMITED COMPANY
http://www.yaxinbio.com; E-mail: zhaozhi@yaxinbio.com; TEL: +86-21-54336592 FAX: +86-21-54336593

Products List

Cat. No.	Product Name	Function	Application	Charaters	Package
RCPB01	Recombinant Carboxypeptidase B	catalyzes lysine, arginine and histidine from C-terminal of polypeptides.	r-insulin production, antibody C-termial identification, sequencing, etc	Special Activity (unit/mg) NLT 170	10mg,100mg,1gr, or bulk.
RPT0201	Recombinant Trypsine (porcine)	endoproteinase, hydrolyzes polypeptides at the carboxyl side of lysine and arginine, comply with USP 2014	r-insulin production, biopharmaceutical process, cell culture	Special Activity (USPU/mg) NLT 3800	10mg,100mg,1gr, or bulk.
SRT0202	Recombinant Trypsine Sequence	endoproteinase, hydrolyzes polypeptides at the carboxyl side of lysine and arginine	peptide mapping, fingerprinting, and sequence analysis	Special Activity (USPU/mg) NLT 6000	20μg, 100μg, 1mg
RHT03	Recombinant Trypsine (human)	endoproteinase, hydrolyzes polypeptides at the carboxyl side of lysine and arginine, comply with USP 2014	biopharmaceutical process,cell culture,cell dissociation, human cell therapy, stem cell, etc.	Special Activity (USPU/mg) NLT 3800	10mg,100mg,1gr, or bulk.
RTS04	Recombinant Trypsine Solution	endoproteinase,hydrolyzes polypeptides at the carboxyl side of lysine and arginine, comply with USP 2014	cell culture,cell dissociation, biopharmaceutical process,etc.	Activity 2000 BAEE unit/ml	10ml,100ml,1L or bulk
RSPA05	Recombinant Protein A	binds to most human and mouse IgG subclasses. can be coupled to solid separation media to purify polyclonal or monoclonal IgG antibodies, can be coupled to a variety of molecules (such as fluorescent molecules, enzyme markers, biotin, colloidal gold and radioactive markers) used in antibody test in the process of Western-blot, ELISA or immunohistochemical tests, ect.		Purity NLT 95%	10mg,100mg,1gr, or bulk.
RLA06	Recombinant Lipase A	catalyzes the hydrolysis of fats and oils with excellent enantioselectivity.(1) Hydrolysis of trans-3-(4-methoxyphenyl) glycidic acid methyl ester [(±)-MPGM] (-)-MPGM.(2) Hydrolysis of (±)-naproxen methyl ester to produce (-)-naproxen. (3)Catalysis of ester substitution reaction, etc.		Activity 1000 unit/gr, 2000 unit/gr	1gr, 10gr, or bulk.
REK08	Enterokinase (EK)	cleaves lysine C-terminal preceded by four aspartic acids: Asp-Asp-Asp-Asp-Lys	delete extra N-terminal fusion protein to gain full recombinant protein	Activity (unit/μl) 10-50	100unit, 500unit, 1ku, or bulk.
RPK09	Recombinant proteinase K	endopeptidase. digest native proteins, thereby inactivating enzymes	used in the process of DNA extraction,etc.	Activity NLT 30	10mg,100mg,1gr, or bulk.
RCT10	Recombinant Chymotrypsine	endoproteinase, hydrolyzes polypeptides at the carboxyl side of aromatic amino acids: Tyr, Phe and Trp.	protein digests for peptide mapping or protein identification by peptide mass fingerprinting or MS/MS spectral matching.	Special Activity (unit/mg) NLT 1000	10mg,100mg or bulk.
V813	V8 (Endoproteinase Glu-C)	endoproteinase, cleaves peptide bonds C-terminal to Glu and Asp	insulin analysis, peptide mapping, fingerprinting, and sequence analysis	Activity (unit/mg) 20	1 unit, 50 μg, 2mg.

重组胰蛋白酶—符合USP2014

- 重组胰蛋白酶的系列产品：

(1) Recombinant trypsin (human 2,PRSS II)
重组人胰蛋白酶I

(2) Recombinant trypsin (human 1,PRSS I)
重组人胰蛋白酶II

(3) Recombinant trypsin (porcine, RPT)
重组猪胰蛋白酶

(4) Recombinant trypsin (bovine, RBT)
重组牛胰蛋白酶

重组生产，无动物源性，彻底改善我国疫苗企业生产的动物源性问题。从一个重要途径上避免了人畜共患病的传播。

应用于疫苗生产，重组蛋白生产、免疫治疗等领域。

Main Products

- **Manufacturer of AOF recombinant enzymes for recombinant human insulin**

the main products in this field:

(1). Recombinant Trypsin;
(2). Recombinant Carboxypeptidase B;
(3). V8 for Insulin Detection;
(4). Recombinant Trypsin Inhibitor (Aprotinin)
(5). Recombinant endoproteinase Lys/Arg-Arg

Main Products

- **Other AOF enzymes and proteins**

(1). Recombinant Enterokinase
(large scale)
(2). Recombinant Protein A
(alkaline stable, for antibody purification)
(3). Recombinant human chymotrypsin

…….

自主知识产权


证书号第1077877号
发明专利
发明名称：一种高稳定性的重组胰蛋白酶的
发明人：冯矗;赵致
专利号：ZL 2009 1 0055493.8
专利申请日：2009年07月28日
专利权人：上海雅心生物技术有限公司
授权公告日：2012年11月14日
局长
证书号第1077867号
发明专利
发明名称：一种高稳定性的重组羧肽酶B的
发明人：冯矗;赵致
专利号：ZL 2009 1 0055492.3
专利申请日：2009年07月28日
专利权人：上海雅心生物技术有限公司
授权公告日：2012年11月14日
局长
证书号第1077877号
发明专利
发明名称：一种高稳定性的重组胰蛋白酶的生产
发明人：冯矗;赵致
专利号：ZL 2009 1 0055493.8
专利申请日：2009年07月28日
专利权人：上海雅心生物技术有限公司
授权公告日：2012年11月14日
局长
证书号第1714571号
发明专利证书
发明名称：高稳定性的具抗体结合能力的重组蛋白A及其生产
发明人：李素霞;赵致;冯矗
专利号：ZL 2010 1 0614100.5
专利申请日：2010年12月30日
专利权人：上海雅心生物技术有限公司
授权公告日：2015年07月01日
局长
申长雨
2015年07月01日

Thank you!