CHROMOGENIC SUBSTRATE TECHNOLOGY

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What is a chromogenic substrate?

- A peptide linked to a chromophore
- The peptide is formed by 3-5 residues
- The chromophore is p-nitroaniline (p-NA)
- The residues can be natural amino acids or chemically modified amino acids
- The sequence of the residues mimics the sequence of the natural substrate
- The hydrolysis of the substrate causes the release of pNA (yellow colored compound)

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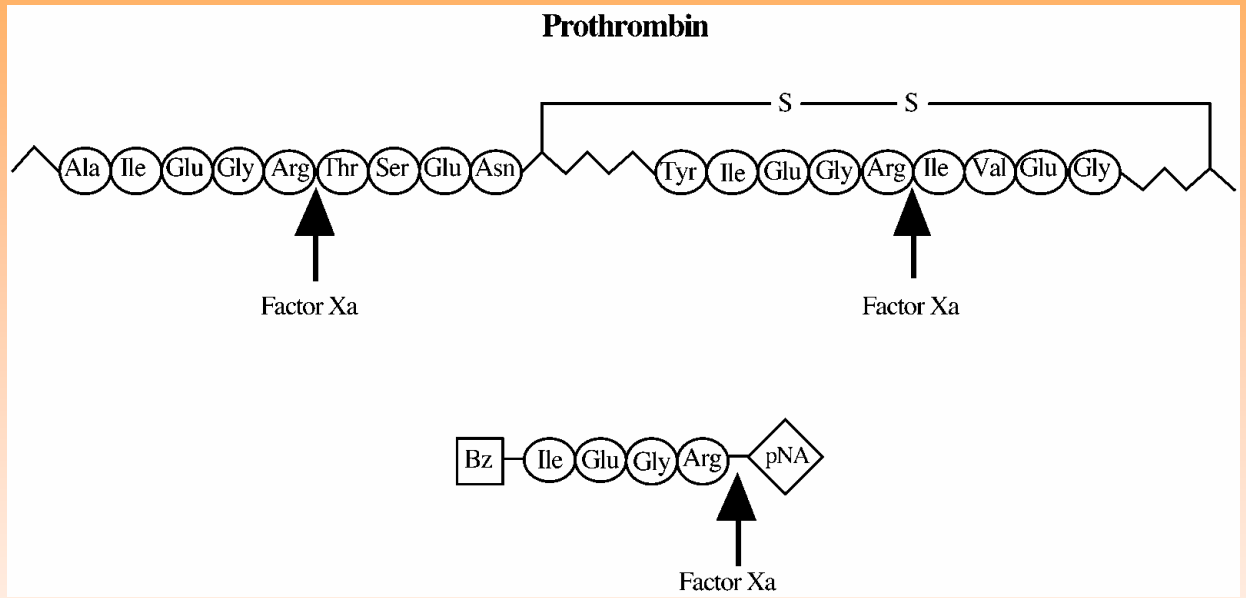
Chemical structure



Prothrombin, the natural substrate of FXa, is cleaved by FXa at two positions, each proceeded by the same four amino acid sequence. FXa activity can be determined by the chromogenic substrate S-2222 which is composed of the same amino acids coupled to a chromophore

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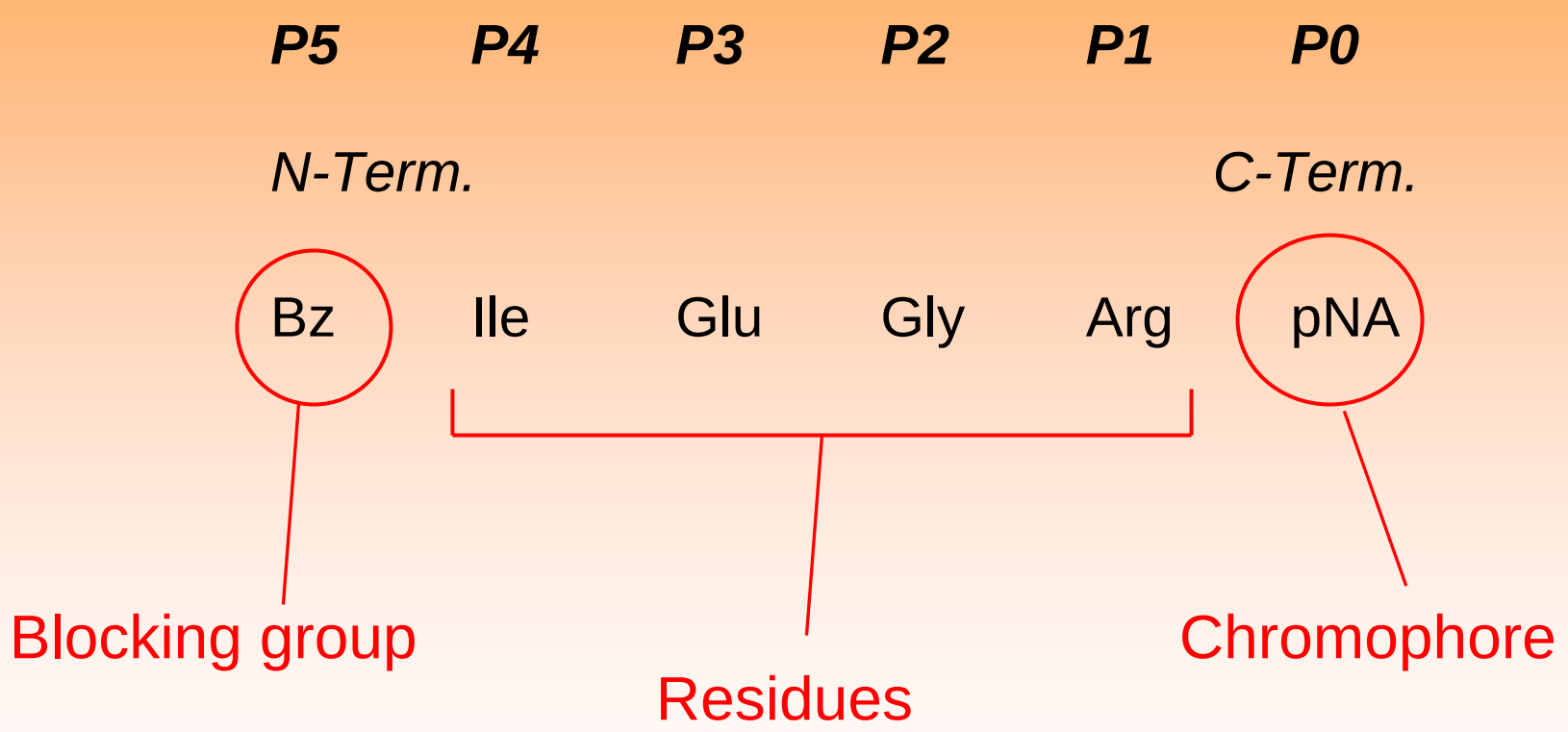
Chemical structure



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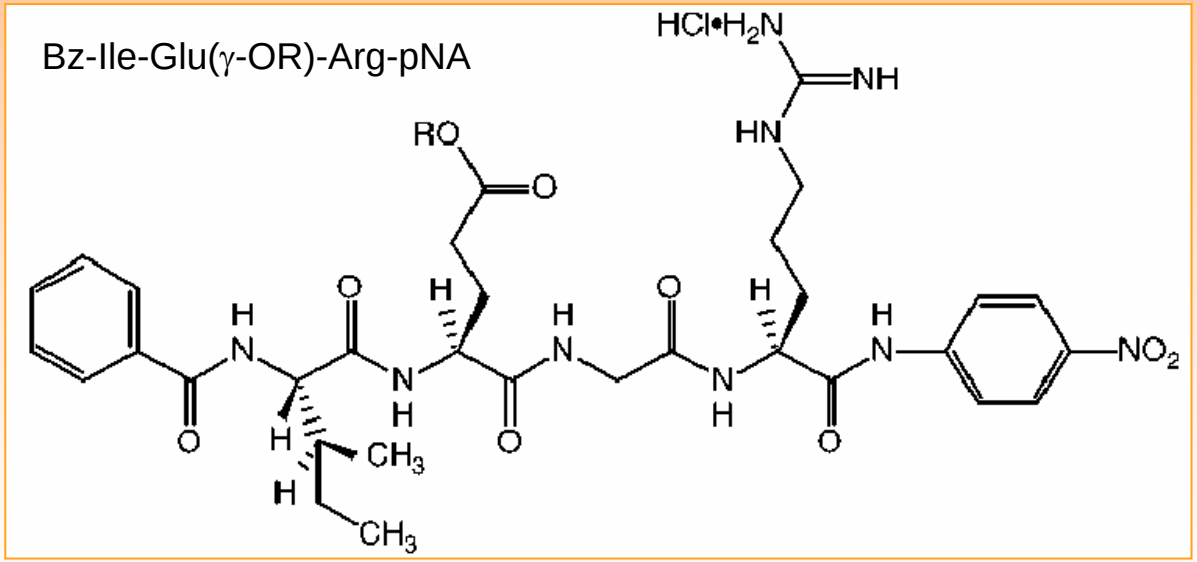
Chemical structure

S-2222: a substrate specific for FXa



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Enzymes

- Proteins that catalyze chemical reactions
- They exerts its catalytic activity upon substrates
- Proteolytic enzymes act on their natural substrates, proteins, by hydrolyzing one or more peptide bond(s)
- The hydrolyzing process is usually highly specific as only peptide bonds adjacent to certain amino acids are cleaved

Classes of proteases

Name	***Active site***
Serine proteases	Ser His Asp*
Cystein proteases	Cys His Asp*
Aspartic proteases	Asp Asp
Metallo proteases	His His Zn^{2+}

**Asp not always present*

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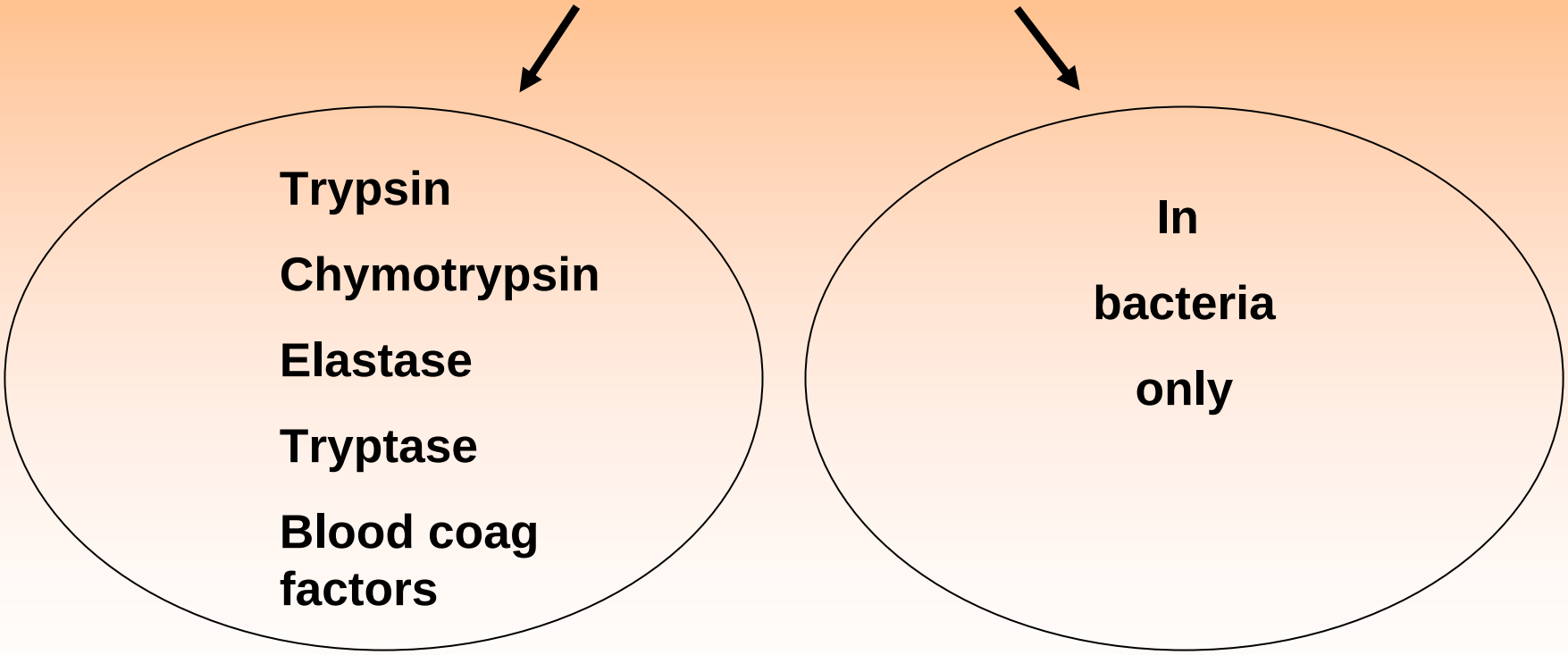
Serine proteases

Two groups: Trypsins and Subtilisin



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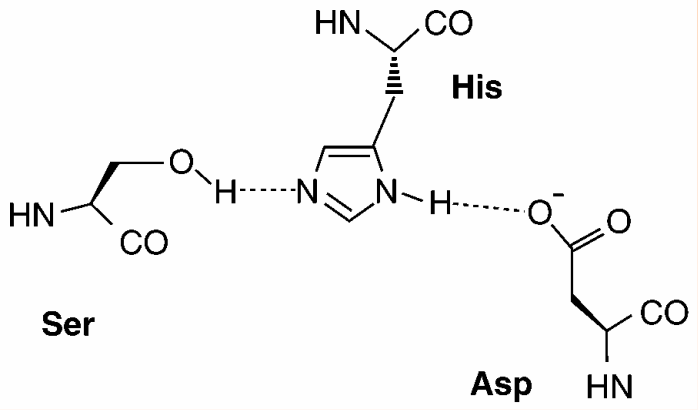
Trypsins

The Trypsin family is classified according to the type of amino acid (a.a.) that occurs at the preferred cleavage site:

Enzyme	***Cleavage site***
Elastase	hydrophobic a.a.
Chymotrypsin	aromatic a.a.
Others	basic a.a. (Arg or Lys)

The catalytic site

- The reaction is the result of the interaction between the substrate and the catalytic site
- The catalytic site is known as the catalytic triad



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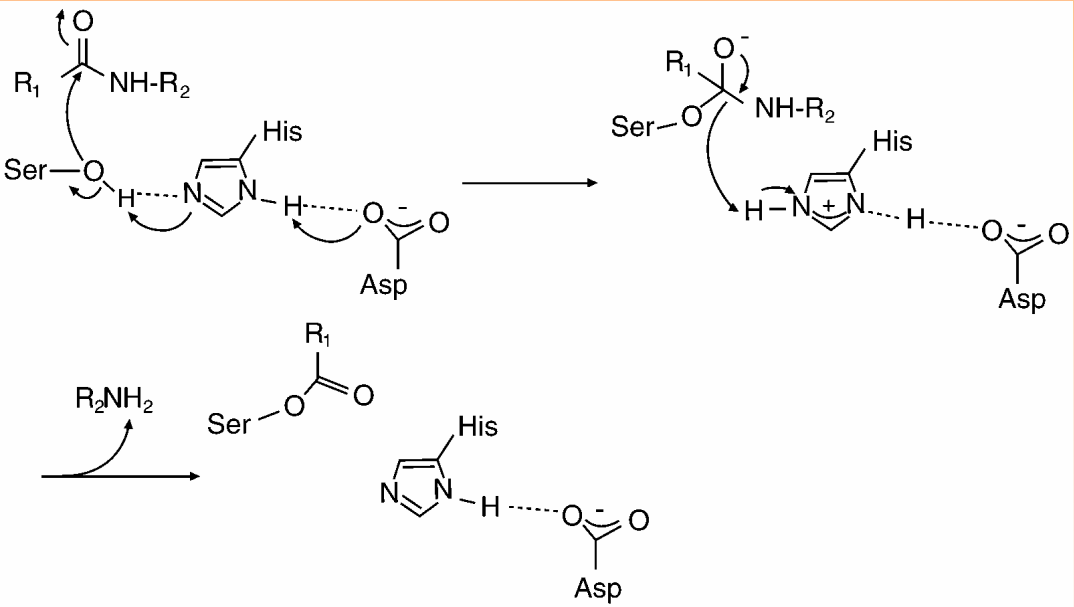
The proteolytic reaction

Formation of an acyl-enzyme intermediate



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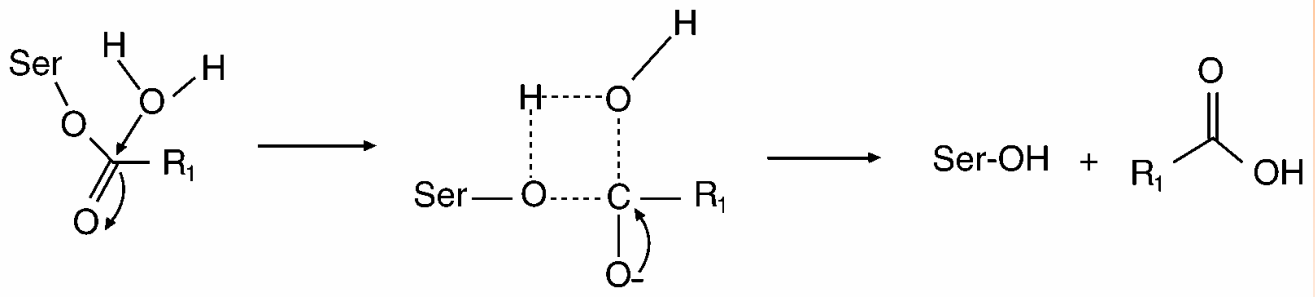
The proteolytic reaction

Hydrolysis of the acyl-enzyme intermediate



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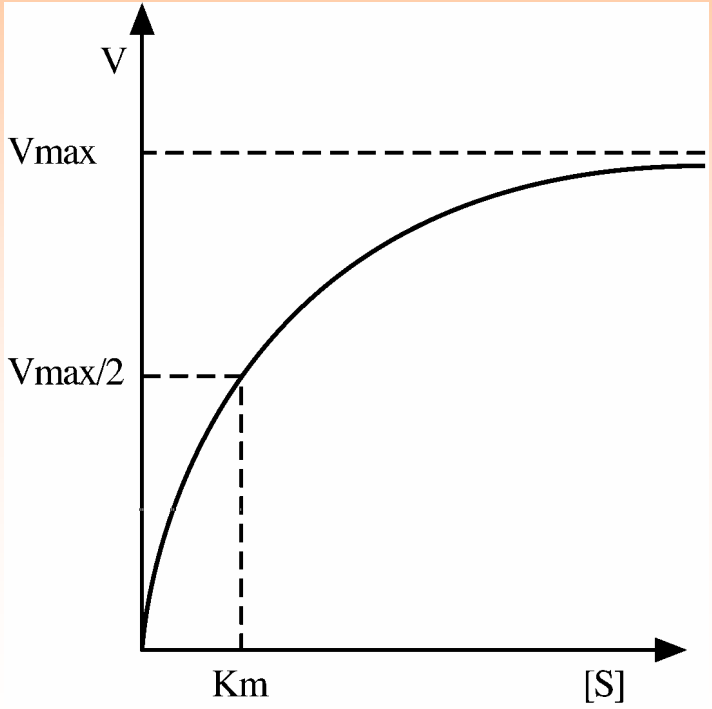
Enzyme kinetics

$$E + S \underset{K2}{\overset{K1}{\rightleftharpoons}} ES \xrightarrow{K3} E + P$$



$$V = Vmax \frac{[S]}{[S] + Km}$$

$$Km = \frac{k_2 + k_3}{k_1}$$

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Enzyme kinetics

- kcat is the turnover number and corresponds to k_3. It is the maximal number of substrate molecules that can be converted to product per time unit

- Km corresponds to the concentration of substrate which gives a reaction rate of Vmax/2.

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Enzyme units

The enzymatic activity is defined in two ways:

- By comparison with the activity of a standard preparation, where the units are defined by WHO, NIH etc…
- By measuring the amount of substrate split, or the product formed per time unit

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Enzyme activity: calculation

- 1nkat = 1 x 10^{-9} mol product released per sec
- pNA has a molar absorptivity of 9600 mol^{-1} L
- A general chromogenic method can be summarized as follows:

Compound	Volume (µL)
Buffer	v1
Sample	v2
Substrate	v3
A) Initial rate method: reading at 405 nm and determination of ΔA/min	
B) Acid stopped method: incubation (t) and addition of Acetic acid	
Acetic acid	v4

nkat/mL = 1.74 x V/v2 x ΔA/min (Initial rate method)

nkat/mL = 1.74 x V/(v2 x t) x A (Acid stopped method)

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Historical background

- The application of chromogenic substrates in hemostasis began in the early 1970s
- BAPNA was the first chromogenic substrate for serine proteases but with poor selectivity
- S-2160 was the first chromogenic thrombin substrate
- Among the 500 pNA peptides synthesized, 24 have been found having the best specificity and reactivity towards the enzymes studied
- Now, our product range covers quite extensively the multiple needs of the customers

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Application of the chromogenic technology:

Antithrombin

Heparin

Protein C

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Antithrombin

Antithrombin is the major thrombin inhibitor, accounting for approximately 80% of the thrombin inhibitory activity in plasma.

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Thrombin inhibition

- Inhibitors
  - Antithrombin
  - a2-macroglobulin
  - Trypsin inhibitor
  - Heparin cofactor II
- Thrombomodulin
  - Turns thrombin into a protein C activator

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Antithrombin - the protein

- 58 KDa single-chain plasma glycoprotein
- Synthesised in the liver
- Plasma concentration 150 μg/ml (2.5 μM)
- Half-life 3 days

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Antithrombin - the inhibitor

- Antithrombin inhibits thrombin, FIXa, FXa, FXIa, FXIIa and the complement enzyme C1.
- Antithrombin forms a 1:1 complex with the inhibited protease.
- The inhibition is enhanced by heparan sulphate, a heparin like substance on the endothelial cells, lining the blood vessels.
- Binding of heparan sulphate to antithrombin induces a conformational change in the antithrombin molecule at the reaction site. This facilitates its reaction with the enzyme.

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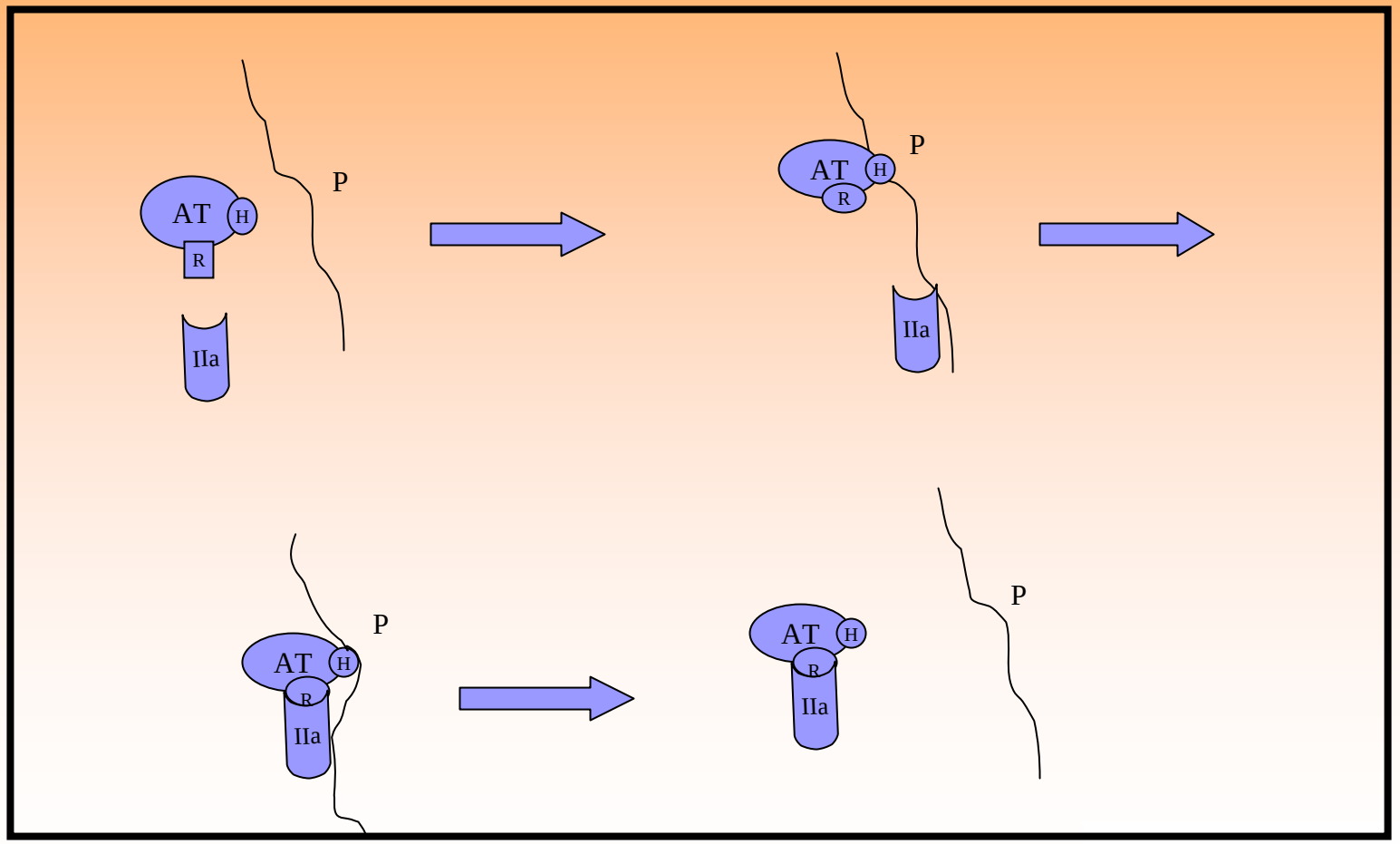
Thrombin inhibition catalysed by heparin



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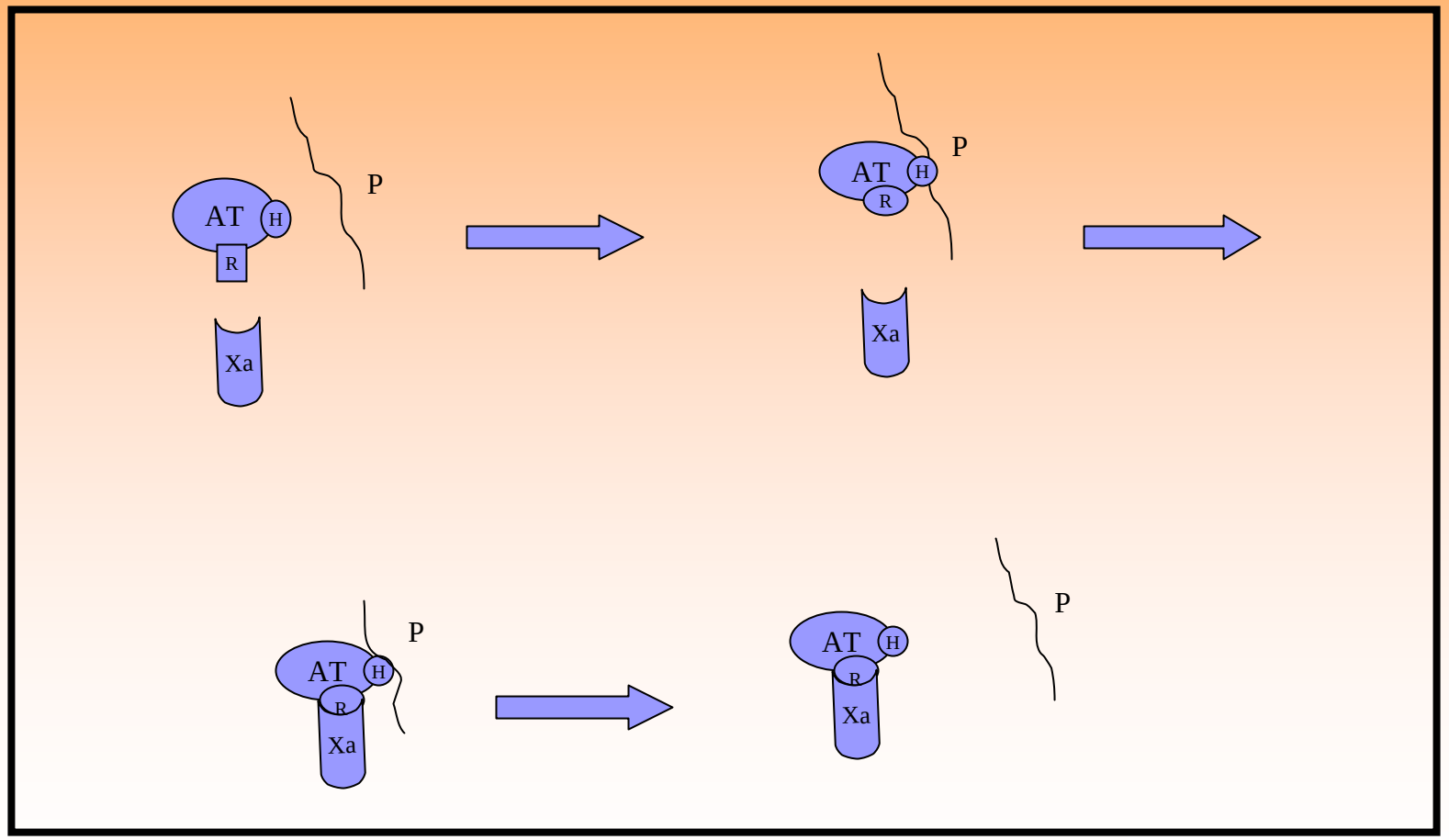
FXa inhibition catalysed by heparin



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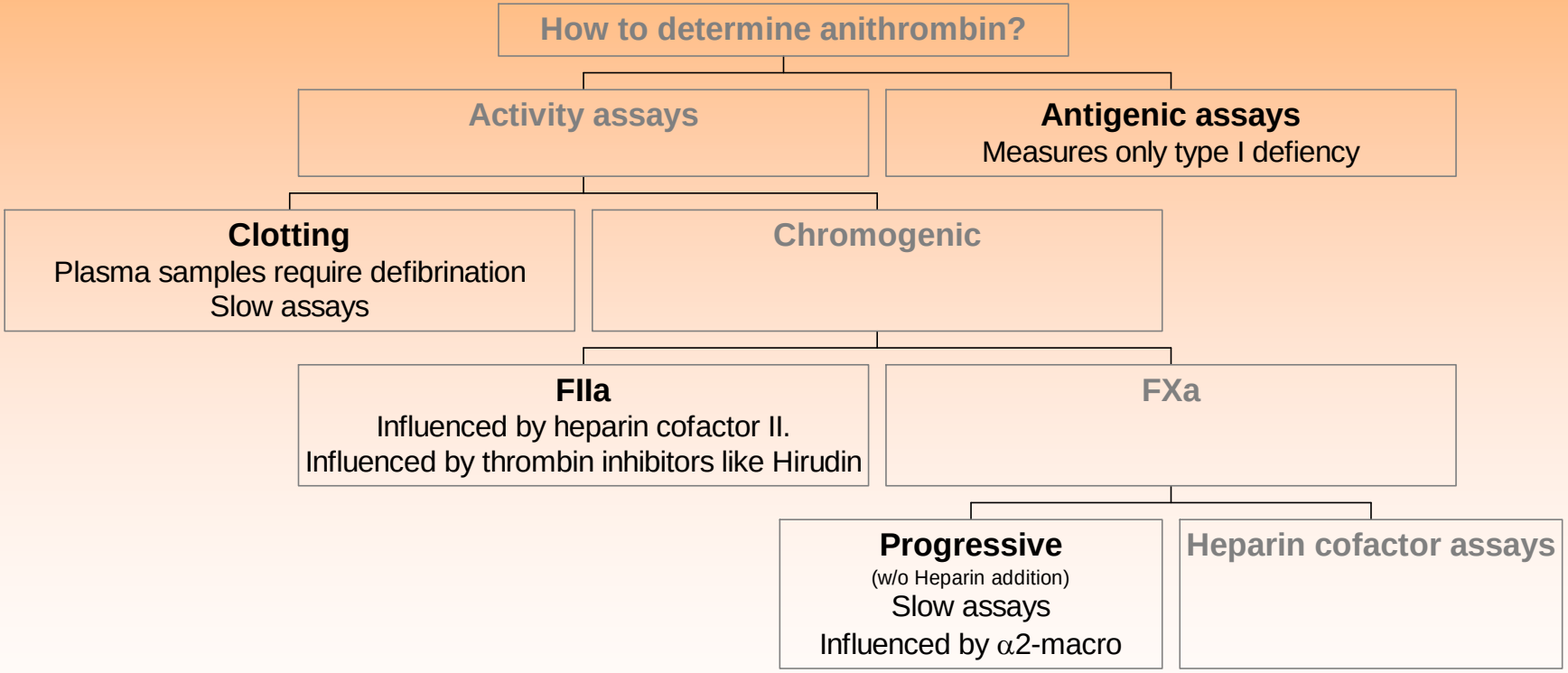
Antithrombin monitoring



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Antithrombin monitoring chromogenic activity assays

Chromogenic heparin cofactor activity assays:

- The sample is incubated with heparin and an excess amount of thrombin or FXa. The residual thrombin or FXa then cleaves a chromogenic substrate

$$AT + Heparin \longrightarrow [AT*Heparin]$$

$$[AT*Heparin] + FXa_{(excess)} \longrightarrow [AT\text{-}FXa\text{-}Heparin] + FXa_{(residual)}$$

$$Chromogenic\ substrate \xrightarrow{FXa_{(residual)}} Peptide + pNA$$

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Antithrombin: anti-FXa assay

Sample/Standard dilution:	25 µl sample+
	3000 µl saline

Procedure	**Volumes**
Diluted sample/standard	50 µl
Factor Xa (2.9 nkat/ml in Hep Buffer)	50 µl
Incubate at 37°C	90 sec
S-2765 (0.8 mg/ml)	50 µl

Read ΔA/min at 405 nm for rate method or add 50 µl Acetic acid after 90 sec incubation for end-point method.

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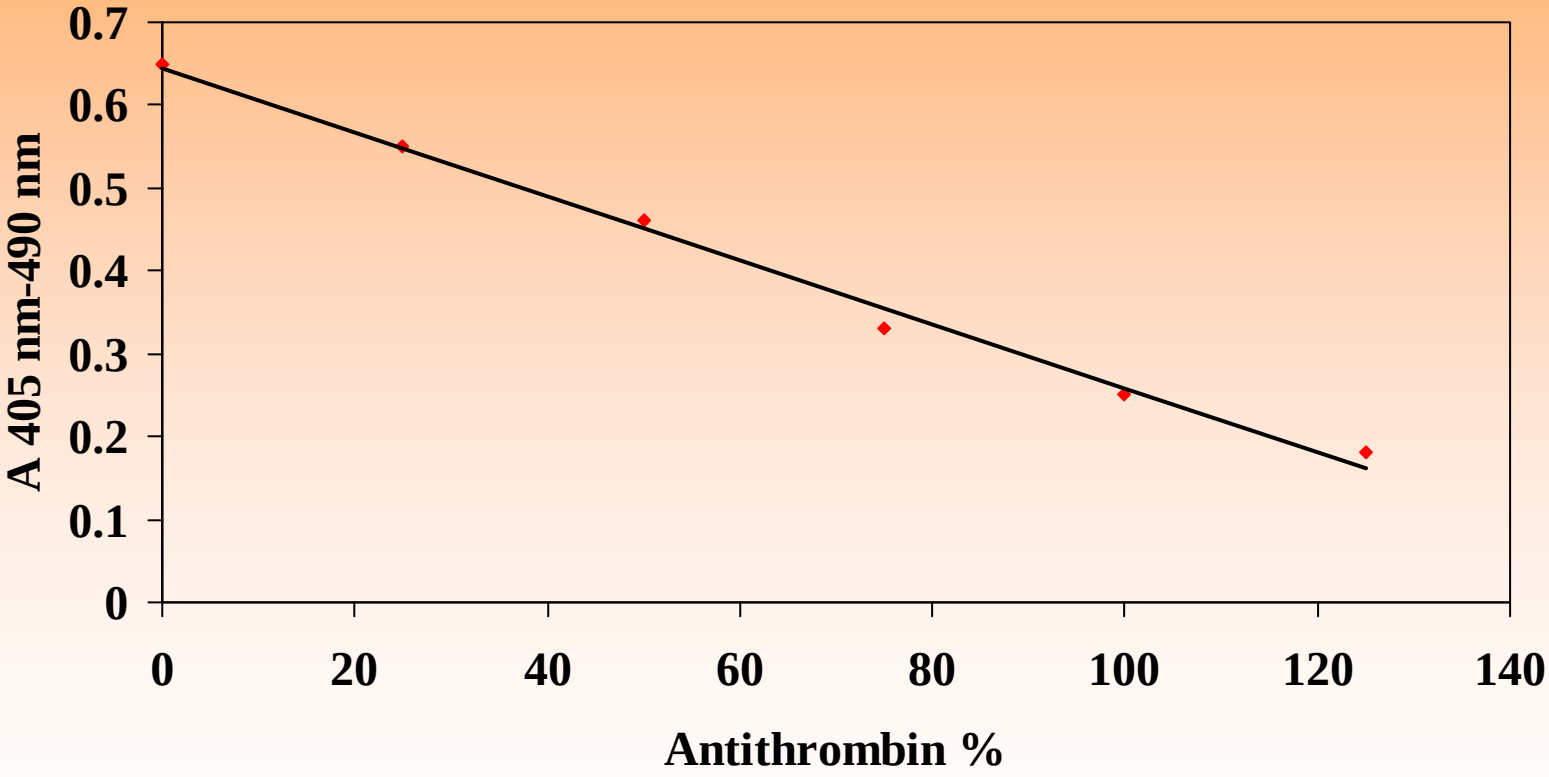
Antithrombin: anti-FXa assay



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Heparin

- Heparin is a heterogeneous mixture of unbranced polysaccharide chains
- Alternating monosaccharide units of L-iduronic acid and D-glucosamine
- The molecule size in the natural extract is 2 to 40 Kda
- One third of the polysaccharide chains contain a specific antithrombin binding pentasaccharide sequence

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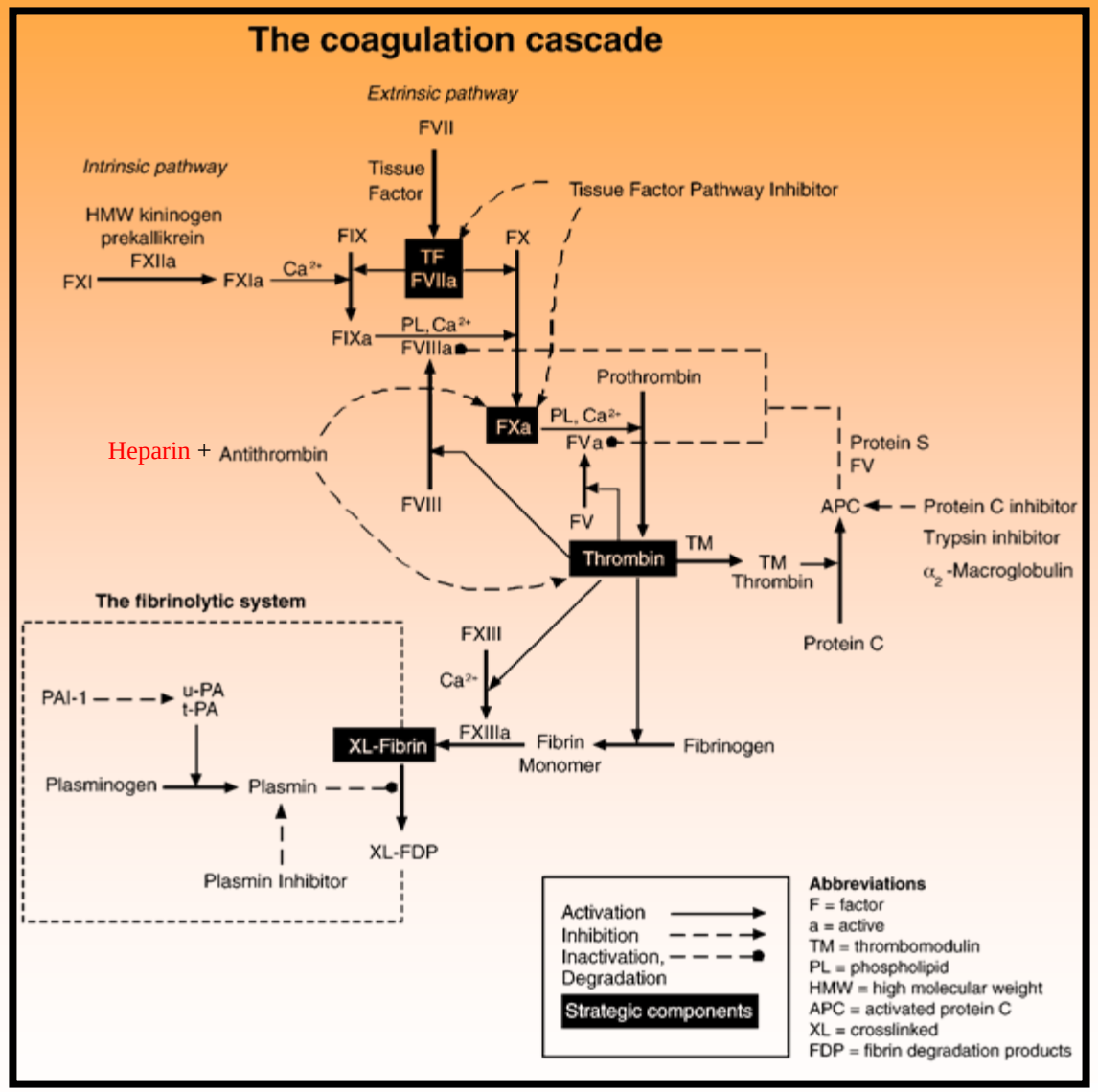

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Heparin

Mechanism of action

- Heparin exerts parts of its anticoagulant activity through interaction with antithrombin
- Antithrombin binds specifically to a pentasaccharide in heparin
- Binding to heparin induces a conformational change in the antithrombin, which accelerate the enzyme inhibition

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Heparin: chromogenic methods

- Anti-FXa activity:

$$AT + Heparin \longrightarrow [AT*Heparin]$$

$$[AT*Heparin] + FXa_{(excess)} \longrightarrow [AT\text{-}FXa\text{-}Heparin] + FXa_{(residual)}$$

$$\text{Chromogenic substrate} \xrightarrow{FXa_{(residual)}} \text{Peptide} + pNA$$

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Heparin: chromogenic methods

- Anti-FIIa activity:

$$AT + \text{Heparin} \longrightarrow [AT^{*}\text{Heparin}]$$

$$[AT^{*}\text{Heparin}] + FIIa_{(excess)} \longrightarrow [\text{Heparin-AT-FIIa}] + FIIa_{(residual)}$$

$$\text{Chromogenic substrate} \xrightarrow{FIIa_{(residual)}} \text{Peptide} + \text{pNA}$$

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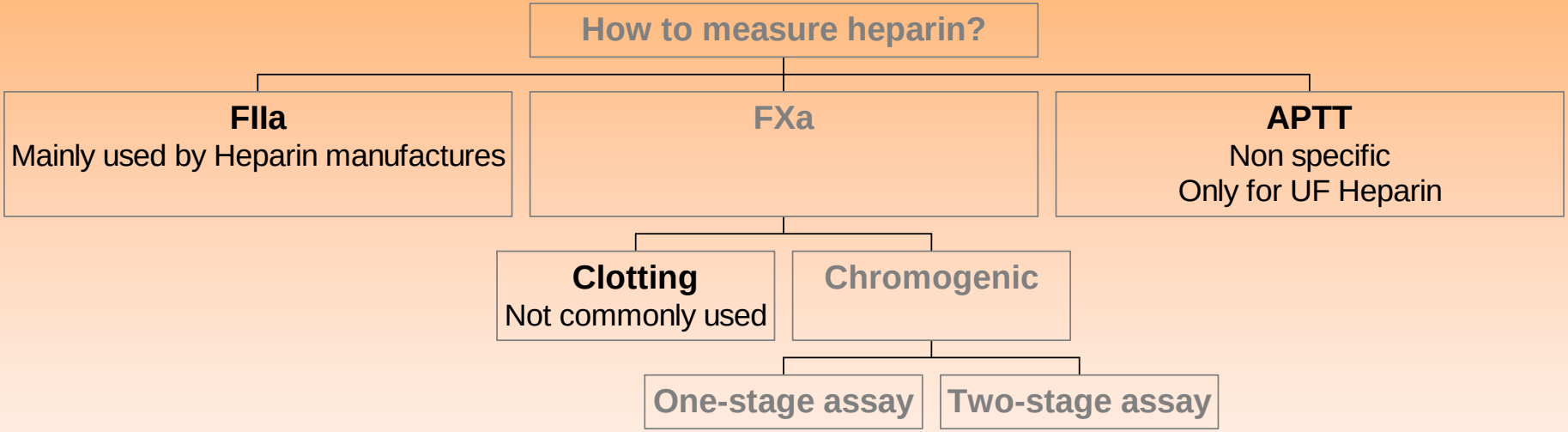
Heparin measurements



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Heparin: one-stage and two-stage

One-stage (CM Heparin)

Sample/standard Dilution:

100 µl sample +

300 µl water

Diluted sample	50 µl
S-2732 (3 mg/ml)	50 µl
FXa (7 nkat/ml)	50 µl
Incubate at 37°C	*120 sec*
Acetic acid	50 µl
Read at 405 nm	

Two-stage (CT Heparin)

Sample/Standard Dilution:

100 µl sample+

100 µl AT (1 IU/ml) +

800 µl Buffer

Diluted sample	200 µl
FXa (7.1 nkat/ml)	100 µl
Incubate at 37°C	*30 sec*
S-2222 (0.75 mg/ml)	200 µl
Incubate at 37°C	*180 sec*
Acetic acid	300 µl
Read at 405 nm	

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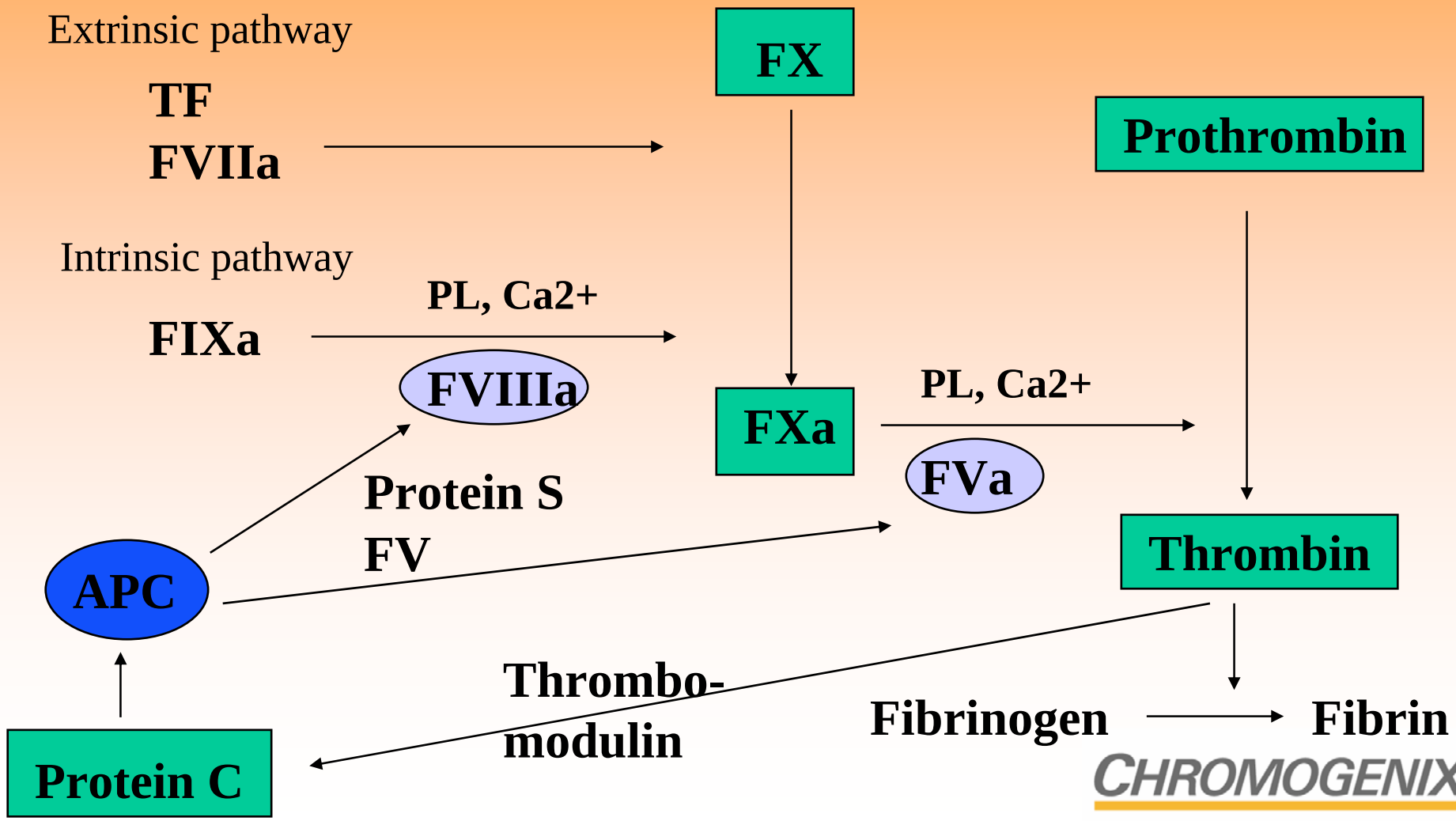
Protein C in the coagulation system



Protein C - an anticoagulant

Vitamin K dependent proteins:

Anticoagulants: protein C and protein S

Procoagulants: Factor II, VII, IX and X

Synthesised in the liver

Glu ⟶ Gla (glutamic acid residues are converted to gamma-carboxyglutamic acid)

The Gla domain binds calcium ions which form a bridge to the phospholipid surfaces on platelets and endothelial cells.

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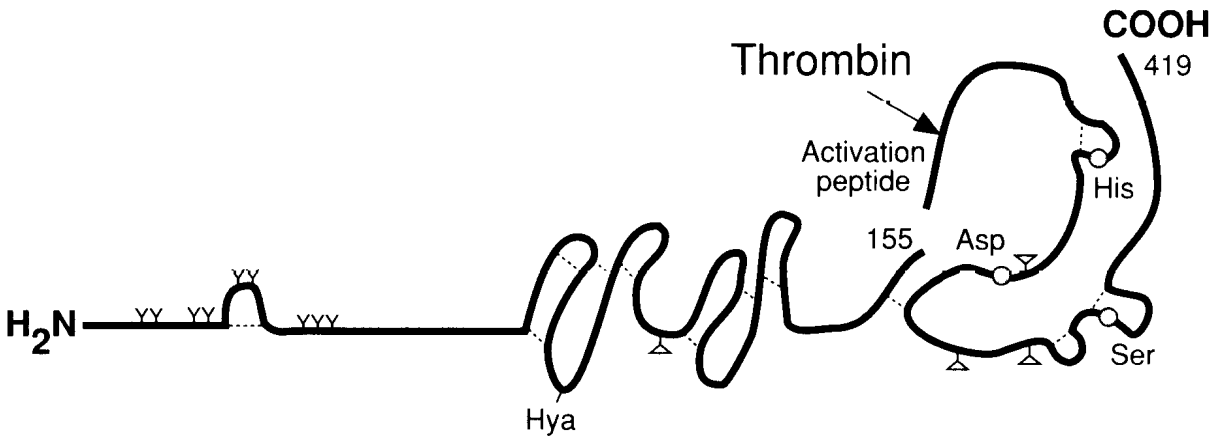
Protein C-the structure



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Protein C- the function

Protein C inhibits coagulation through inactivation of FVIIIa and FVa

Protein C potentiates the fibrinolytic system by inhibiting PAI-1, the major inhibitor of fibrinolysis.

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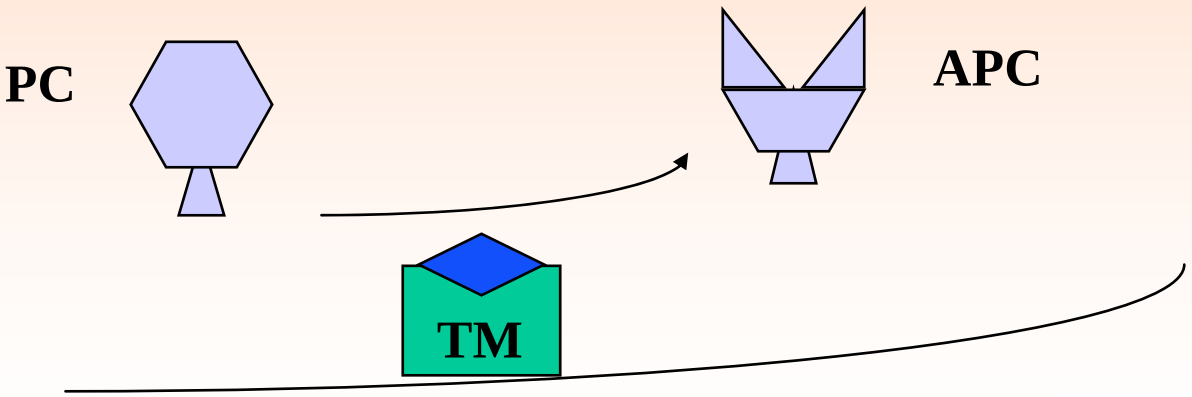
Protein C activation

Protein C is activated (to APC) by thrombin.
A small peptide is removed.
Activation by thrombin alone is slow. The complex thrombin-thrombomodulin activates protein C 20 000 times faster.



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Protein C activation

Thrombomodulin (TM) is a membrane protein present on the endothelium.

TM has the following effects on thrombin:

- Increases the rate by which thrombin activates protein C
- Removes the procoagulant properties of thrombin
- Accelerates the thrombin-antithrombin reaction

APC cofactors

APC has two known cofactors: Protein S and Factor V.

Protein S:

Protein S enhances binding of APC to the phospholipid of platelets and endothelial cells.

Only free protein S has a APC cofactor function. 60% of protein S is bound to C4bBP.

Factor V

Factor V together with Protein S makes APC degrade FVIIIa and FVa more effectively.

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Protein C assays - principles: Chromogenic assays

Chromogenic assays

Utilise specific protein C activator (Protac) for the activation of protein C

The activated protein C cleaves a chromogenic substrate.

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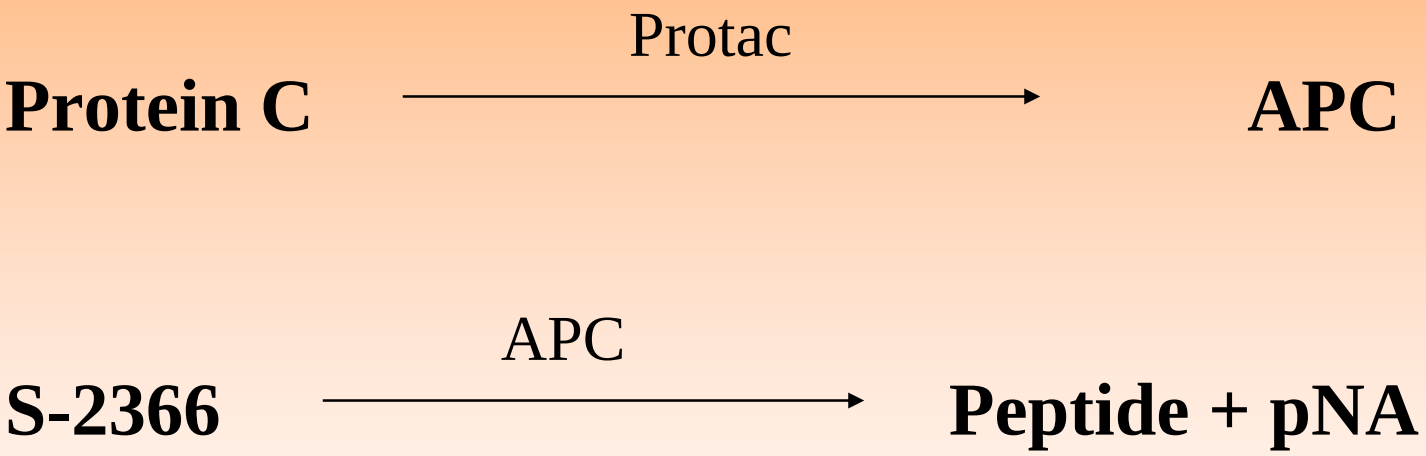
Coamatic Protein C - the principle



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Back to the chromogenic substrates……

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Product range:

- **S-2222™** ▸ ***Factor Xa***
- **S-2238™** ▸ ***Thrombin***
- **S-2251™** ▸ ***Plasmin and Streptokinase-activated Plasminogen***
- **S-2266™** ▸ ***Glandular Kallikrein and Factor XIa***
- **S-2288™** ▸ ***t-PA; other proteases***
- **S-2302™** ▸ ***plasma kallikrein; Factor XIa; Factor XIIa***
- **S-2314™** ▸ ***C1s***
- **S-2366™** ▸ ***activated protein C; factor XIa***
- **S-2390™** ▸ ***Plasmin***
- **S-2403™** ▸ ***Plasmin and Streptokinase-activated Plasminogen***
- **S-2423™** ▸ ***Endotoxin determination***
- **S-2444™** ▸ ***Urokinase***
- **S-2484™** ▸ ***Granulocyte elastase***
- **S-2586™** ▸ ***Chymotrypsin***
- **S-2765™** ▸ ***Factor Xa***
- **S-2772™** ▸ ***Factor Xa***

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Beyond the chemical synthesis

- Scientific support
- Well characterized substrates, i.e. kinetic tables
- Research Methods
- New Methods
- Pharmacopoeia abstracts
- Complementary products: Bioreagents

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Substrate selectivity

Enzyme	Substrate	Thrombin (B)	FXa (B)	FXIa (H)	APC (H)	Plasmin (H)	Single chain t-PA (H)	Plasma Kallikrein (H)	C1s (H)	Buffer 50 mM Tris HCl pH	Buffer 50 mM Tris HCl NaCl (mM)	Substrate conc 2xKm (mM)
Thrombin	S-2238	100 (0.11)	5	5	40	5	5	60	2	8.3	130	0.20*
	S-2366	100 (0.14)	5	35	80	70	3	130	2			0.29
	S-2846	100 (0.078)	3	5	30	5	1	30	2			0.090
FXa	S-2222	1	100 (0.34)	2	0	2	2	5	1	8.3	130	0.80
	S-2337	1	100 (0.37)	1	0	2	2	3	1			0.60
	S-2732	1	100 (0.51)	1	0	2	1	3	0			0.70
	S-2765	0	100 (0.61)	1	2	1	5	15	1			0.22
	S-2767	1	100 (0.53)	1	2	1	5	3	1			0.44
	S-2772	1	100 (0.32)	1	2	1	4	4	5			1.4
	S-2782	0	100 (0.63)	2	1	1	10	10	1			0.30
	S-2787	0	100 (0.45)	1	1	1	10	10	2			0.28
FXIa	S-2288	130	290	100 (0.077)	–	–	–	760	75	8.3	130	1.8
	S-2366	150	35	100 (0.14)	–	–	–	360	10			2.4
APC	S-2288	80	30	25	100 (0.13)	15	–	170	–	8.3**	–	0.32
	S-2366	75	4	30	100 (0.19)	60	–	110	–			0.40
	S-2846	70	4	15	100 (0.16)	20	–	80	–			0.70

B=bovine H=human

** Substrate conc 20×Km*
*** Buffer see table 2*

The Chromogenix catalogue includes a section which shows the cross-reactivity of the substrates with the different enzymes tested

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Kinetic data

	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/(mM•s)) •10^{-3}	Enzyme concentration (mg/L) for ΔA/min=0.05 at [S]=2•K_m
Thrombin, human				
Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl				
S-2238	0.0070	180	26	0.03
S-2366	0.15	330	2.2	0.02
S-2846	0.043	190	4.4	0.03
Thrombin, bovine				
Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl				
S-2238	0.010	200	20	0.02
S-2366	0.15	295	2.0	0.02
S-2846	0.045	200	4.4	0.03
FXa, human				
Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl, 0.5% BSA				
S-2222	1.1	100	0.090	0.06
S-2337	0.67	110	0.16	0.05
S-2732	1.5	230	0.15	0.03
S-2765	0.26	240	0.92	0.02
S-2767	0.60	210	0.35	0.03
S-2772	1.5	120	0.080	0.05
S-2782	0.29	210	0.72	0.03
S-2787	0.38	170	0.45	0.03
FXa, bovine				
Buffer: 50 mM Tris HCl, pH 8.3, 130 mM NaCl				
S-2222	0.40	100	0.25	0.06
S-2337	0.30	110	0.37	0.05
S-2732	0.35	130	0.37	0.05
S-2765	0.11	195	1.8	0.03
S-2767	0.22	160	0.73	0.04
S-2772	0.70	100	0.14	0.06
S-2782	0.15	190	1.3	0.03
S-2787	0.14	120	0.86	0.05

The Chromogenix catalogue includes a section which shows the kinetic data of the substrates toward the different enzymes tested

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The 25 mg Substrates

The substrates are packaged in vials containing 25 mg lyophilized substrate and mannitol as a bulking agent

The insert sheet is in three languages and contains the following information:

Chemical Name

Formula

Solubility

Stability

Kinetic data

Standardization

Applications

References

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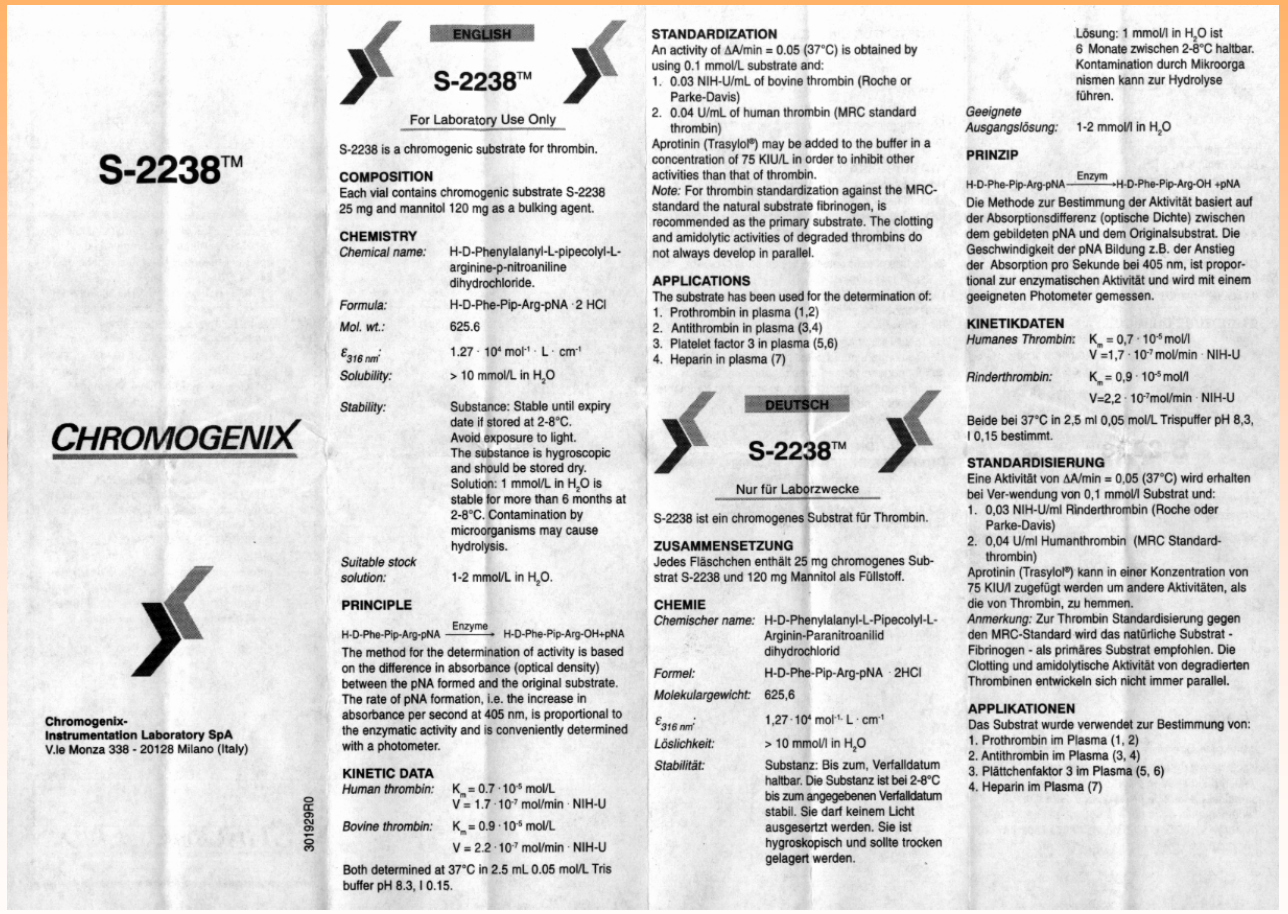
The 25 mg Substrates



S-2238™

CHROMOGENIX

Chromogenix-
Instrumentation Laboratory SpA
V.le Monza 338 - 20128 Milano (Italy)

301929R0

ENGLISH

S-2238™

For Laboratory Use Only

S-2238 is a chromogenic substrate for thrombin.

COMPOSITION
Each vial contains chromogenic substrate S-2238 25 mg and mannitol 120 mg as a bulking agent.

CHEMISTRY

Chemical name: H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroaniline dihydrochloride.

Formula: H-D-Phe-Pip-Arg-pNA · 2 HCl

Mol. wt.: 625.6

$\varepsilon_{316\ nm}$: $1.27 \cdot 10^4\ mol^{-1} \cdot L \cdot cm^{-1}$

Solubility: > 10 mmol/L in H_2O

Stability: Substance: Stable until expiry date if stored at 2-8°C. Avoid exposure to light. The substance is hygroscopic and should be stored dry. Solution: 1 mmol/L in H_2O is stable for more than 6 months at 2-8°C. Contamination by microorganisms may cause hydrolysis.

Suitable stock solution: 1-2 mmol/L in H_2O.

PRINCIPLE

H-D-Phe-Pip-Arg-pNA $\xrightarrow{\text{Enzyme}}$ H-D-Phe-Pip-Arg-OH+pNA

The method for the determination of activity is based on the difference in absorbance (optical density) between the pNA formed and the original substrate. The rate of pNA formation, i.e. the increase in absorbance per second at 405 nm, is proportional to the enzymatic activity and is conveniently determined with a photometer.

KINETIC DATA

Human thrombin: $K_m = 0.7 \cdot 10^{-5}$ mol/L
$V = 1.7 \cdot 10^{-7}$ mol/min · NIH-U

Bovine thrombin: $K_m = 0.9 \cdot 10^{-5}$ mol/L
$V = 2.2 \cdot 10^{-7}$ mol/min · NIH-U

Both determined at 37°C in 2.5 mL 0.05 mol/L Tris buffer pH 8.3, I 0.15.

STANDARDIZATION
An activity of ΔA/min = 0.05 (37°C) is obtained by using 0.1 mmol/L substrate and:
1. 0.03 NIH-U/mL of bovine thrombin (Roche or Parke-Davis)
2. 0.04 U/mL of human thrombin (MRC standard thrombin)

Aprotinin (Trasylol®) may be added to the buffer in a concentration of 75 KIU/L in order to inhibit other activities than that of thrombin.
Note: For thrombin standardization against the MRC-standard the natural substrate fibrinogen, is recommended as the primary substrate. The clotting and amidolytic activities of degraded thrombins do not always develop in parallel.

APPLICATIONS
The substrate has been used for the determination of:
1. Prothrombin in plasma (1,2)
2. Antithrombin in plasma (3,4)
3. Platelet factor 3 in plasma (5,6)
4. Heparin in plasma (7)

DEUTSCH

S-2238™

Nur für Laborzwecke

S-2238 ist ein chromogenes Substrat für Thrombin.

ZUSAMMENSETZUNG
Jedes Fläschchen enthält 25 mg chromogenes Substrat S-2238 und 120 mg Mannitol als Füllstoff.

CHEMIE

Chemischer name: H-D-Phenylalanyl-L-Pipecolyl-L-Arginin-Paranitroanilid dihydrochlorid

Formel: H-D-Phe-Pip-Arg-pNA · 2HCl

Molekulargewicht: 625,6

$\varepsilon_{316\ nm}$: $1,27 \cdot 10^4\ mol^{-1} \cdot L \cdot cm^{-1}$

Löslichkeit: > 10 mmol/l in H_2O

Stabilität: Substanz: Bis zum, Verfalldatum haltbar. Die Substanz ist bei 2-8°C bis zum angegebenen Verfalldatum stabil. Sie darf keinem Licht ausgesetzt werden. Sie ist hygroskopisch und sollte trocken gelagert werden.

Lösung: 1 mmol/l in H_2O ist 6 Monate zwischen 2-8°C haltbar. Kontamination durch Mikroorganismen kann zur Hydrolyse führen.

Geeignete Ausgangslösung: 1-2 mmol/l in H_2O

PRINZIP

H-D-Phe-Pip-Arg-pNA $\xrightarrow{\text{Enzym}}$ H-D-Phe-Pip-Arg-OH +pNA

Die Methode zur Bestimmung der Aktivität basiert auf der Absorptionsdifferenz (optische Dichte) zwischen dem gebildeten pNA und dem Originalsubstrat. Die Geschwindigkeit der pNA Bildung z.B. der Anstieg der Absorption pro Sekunde bei 405 nm, ist proportional zur enzymatischen Aktivität und wird mit einem geeigneten Photometer gemessen.

KINETIKDATEN

Humanes Thrombin: $K_m = 0,7 \cdot 10^{-5}$ mol/l
$V = 1,7 \cdot 10^{-7}$ mol/min · NIH-U

Rinderthrombin: $K_m = 0,9 \cdot 10^{-5}$ mol/l
$V = 2,2 \cdot 10^{-7}$ mol/min · NIH-U

Beide bei 37°C in 2,5 ml 0,05 mol/L Trispuffer pH 8,3, I 0,15 bestimmt.

STANDARDISIERUNG
Eine Aktivität von ΔA/min = 0,05 (37°C) wird erhalten bei Ver-wendung von 0,1 mmol/l Substrat und:
1. 0,03 NIH-U/ml Rinderthrombin (Roche oder Parke-Davis)
2. 0,04 U/ml Humanthrombin (MRC Standard-thrombin)

Aprotinin (Trasylol®) kann in einer Konzentration von 75 KIU/l zugefügt werden um andere Aktivitäten, als die von Thrombin, zu hemmen.
Anmerkung: Zur Thrombin Standardisierung gegen den MRC-Standard wird das natürliche Substrat - Fibrinogen - als primäres Substrat empfohlen. Die Clotting und amidolytische Aktivität von degradierten Thrombinen entwickeln sich nicht immer parallel.

APPLIKATIONEN
Das Substrat wurde verwendet zur Bestimmung von:
1. Prothrombin im Plasma (1, 2)
2. Antithrombin im Plasma (3, 4)
3. Plättchenfaktor 3 im Plasma (5, 6)
4. Heparin im Plasma (7)

CHROMOGENIX

The "bulk" substrates

- All the substrates present in the catalogue can be supplied as bulk material if the amount required is very high (grams)
- The bulk consists of a powder composed by the substrate, without the addition of mannitol
- The bulk is provided in vials, with a vial label and a certificate of analysis

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Substrates Applications: The Chromogenix Kits

Coamatic AT……..	S-2765
Coamatic AT 400……..	S-2772
Coamatic LR AT…….	S-2772
Coamatic Protein C……..	S-2366
Coaset Factor VII……..	S-2765
Coamatic Factor VIII……..	S-2765
Coatest Factor VIII…….	S-2222
Coatest Soluble Fibrin……..	S-2403
Coamatic Heparin……..	S-2732
Coatest LMW Heparin/Heparin……..	S-2732
Coatest Heparin…….	S-2222
Coatest PAI…….	S-2403
Coaset t-PA…….	S-2251
Coamatic Plasmin Inhibitor……..	S-2403
Coamatic Plasminogen…….	S-2403

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Substrates Applications: The Chromogenix Research Methods

Proteolytic Activity……	S-2288
Urokinase……	S-2444
Factor X……..	S-2765
t-PA……	S-2288
Prekallikrein activator (PKA)……	S-2302
Kallikrein-like activity……	S-2302
Kallikrein inhibitor……..	S-2302
Prekallikrein……..	S-2302
Urine Kallikrein……..	S-2266
Granulocyte Elastase……..	S-2484
Trypsin……	S-2222
Chymotrypsin……	S-2586
Antithrombin (FIIa)……	S-2238
Heparin (FIIa)……..	S-2238

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Substrates Applications: The Chromogenix New Methods

Prothrombin Activity……S-2238

Hirudin…… S-2366

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Substrates Applications: Chromogenic methods in quality control - European and U.S. Pharmacopoeia

The European Pharmacopoeia

S-2238	↯	Antithrombin potency LMW-heparin activity (Anti-FIIa) Heparin in factor concentrates
S-2765	↯	LMW-heparin activity (Anti-FXa)
S-2302	↯	PKA in albumin and IgG

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Substrates Applications: Chromogenic methods in quality control - European and U.S. Pharmacopoeia

The U.S. Pharmacopoeia

S-2222	↯	Heparin (anti-FXa activity)
S-2288	↯	Alteplase

FDA recommendations:

S-2302	↯	PKA in albumin and IgG

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