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Processing of Proapolipoprotein AI requires Specific Conformation

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Dedicated to Prof. Dr. Karl Decker on the occasion of his 60th birthday

Summary: Apolipoprotein AI of human high-density lipoproteins is secreted by hepatocytes as a proapolipoprotein with a N-terminal hexapeptide sequence (Arg-His-Phe-Trp-Gln-Gln-) which differs from the prosequence of rat apolipoprotein AI (Trp-Asp-Phe-Trp-Gln-Gln). The two proteins have in common the unusual cleavage site -Gln-Gln-Asp-Glu-. It is hydrolysed by a specific serum proteinase with the release of mature apo AI.

We synthesized a model substrate for the study of the final processing of pro-apo AI by the serum proteinase. It is an undecapeptide embracing the human pro-hexapeptide sequence and the first five N-terminal residues of apo AI, covalently linked to a hydrophilic resin. The N-terminal arginine residue was ^{3}H-labelled.

$$[^3H]\overset{-6}{\text{Arg}}\text{-His-Phe-Trp-Gln-}\overset{-1}{\text{Gln}}\text{-}\overset{+1}{\text{Asp}}\text{-Glu-Pro-Pro-}\overset{+5}{\text{Gln}}\text{-resin}$$

This sequence was not cleaved by human serum under the conditions under which rat serum processes the pro-form of apo AI secreted by rat hepatocytes. Pepsin and chymotrypsin fragmented the undecapeptide at sites characteristic for these proteinases.

We conclude that the proteolytic cleavage at the specific site (-Gln-Gln-Asp-Glu-) requires the correct conformation in addition to the specific amino-acid sequence.

Prozessierung des Proapolipoproteins AI erfordert eine spezifische Konformation

Zusammenfassung: Apolipoprotein AI des menschlichen High-Density-Lipoproteins wird als ein Proapolipoprotein mit einer N-terminalen Hexapeptid-Sequenz Arg-His-Phe-Trp-Gln-Gln- von Hepatozyten in das Plasma sezerniert. Sie unterscheidet sich von der Pro-Sequenz des Rattenapolipoproteins AI, die aus dem Hexapeptid Trp-Asp-Phe-Trp-Gln-Gln besteht. Beiden Proteinen gemeinsam ist die -Gln-Gln-Asp-Glu- Sequenz an der Spaltstelle. Diese ungewöhnliche Sequenz wird durch eine spezifische Serumprotease zum reifen Apolipoprotein AI gespalten.

Wir synthetisierten als ein Modell-Substrat für die Untersuchung dieser Reaktion ein Undecapeptid, das die Pro-Sequenz und die ersten fünf Aminosäuren des N-Terminus des reifen Apo-AI umfaßt und an ein hydrophiles Harz kovalent gebunden ist:

$$[^3H]\overset{-6}{\text{Arg}}\text{-His-Phe-Trp-Gln-}\overset{-1}{\text{Gln}}\text{-}\overset{+1}{\text{Asp}}\text{-Glu-Pro-Pro-}\overset{+5}{\text{Gln}}\text{-Harz}$$

Der N-terminale Argininrest des an eine Festphase gebundenen Undecapeptids wurde ^{3}H-markiert, um die proteolytische Spaltung radiochemisch verfolgen zu können. Menschliches Serum spaltete unter den Bedingungen, unter denen von Ratten-Hepatozyten sezerniertes Proapo-AI gespalten wird, nicht. Dagegen spalteten Pepsin und Chymotrypsin das gekoppelte Undecapeptid an den für diese Proteinasen charakteristischen Stellen.

Abbreviations:
Apo AI and apo AII, mature apolipoproteins AI and AII; HDL, high-density lipoproteins (d = 1.09–1.21 g/m*l*).

Wir schließen daraus, daß zur proteolytischen Spaltung nicht nur die spezifische Aminosäuresequenz, sondern auch eine bestimmte Konformation erforderlich ist.

Key words: Proapolipoprotein AI sequence, secretion and processing by serum proteinase, solid phase peptide synthesis, proteolytic cleavage of resin linked prosequence.

Apolipoproteins, the protein moieties of serum lipoprotein particles, are synthesized either in hepatocytes or enterocytes of the intestinal mucosa, and secreted into the blood stream. Recent studies in this and other laboratories have analysed the pathway of the synthesis, the processing and the secretion of the two main apolipoproteins of HDL in rat and man[1–6]. The primary translation products of apo AI and apo AII mRNAs are prepro-apoproteins. Prepro-apo AI has an N-terminal extension of 24 amino-acid residues, 18 of which are cleaved to produce the intracellularly stable pro-form during the cotranslational translocation into the lumen of the endoplasmic reticulum by the signal peptidase.

Pro-apo AI is secreted into the serum. The hexapeptide sequence at the N-terminus of pro-apo AI is cleaved by a serum proteinase specific for an unusual cleavage site in which Gln-Gln is linked to the N-terminal Asp-Glu of mature apo AI[7,8]. The pro-sequences of apo AI in man and rat differ, but are homologous at the cleavage site.

Similarly the primary translation product of apo AII mRNA carries a 23 amino-acid residue prepro-sequence; 18 of these are regarded as signal peptide and 5 as pro-sequence[3,5,9]. Whereas the pro-apo AI form is totally secreted from mucosa cells or hepatocytes, pro-apo AII is cleaved intracellularly to a large extent (50%) to mature apo AII, and about equal amounts of pro- and mature apo AII are secreted. These data have been obtained from isolated hepatocyte suspension cultures or human hepato-carcinoma derived (Hep G2) cell cultures[5,6,9].

Major sequence data of prepro-apo AI and AII have been obtained by cell-free translation experiments in the presence of a number of labelled amino acids, subsequent purification of the labelled prepro- and pro-forms and automated Edman degradation. The sequences given in Fig. 1a and b were completed by data from recent DNA-sequences of isolated cDNA apo AI and apo AII clones[10–14]. The pro-sequence of apo AII terminates at two Arg-residues, a basic site typical for other pro-proteins, e.g. proalbumin.

Sufficient amounts of pro-apo AI (Fig. 1a) are required for the study of the final processing step, i.e. the cleavage of pro-apo AI to mature apo AI by a serum proteinase specific for the sequence Gln-Gln-Asp-Glu as well as for the study of the properties of this enzyme. We tried to mimic the substrate by an artificial particle consisting of a hydrophilic resin, covalently substituted by a radiolabelled undecapeptide comprising the hexapeptide pro-sequence and the first five amino-acid residues of mature human apo AI. A partial structure is given in Fig. 1c. This substrate was used to probe the serum for the specific protease activity.

Whereas no release of ^{3}H-activity was observed with serum, other proteinases such as trypsin

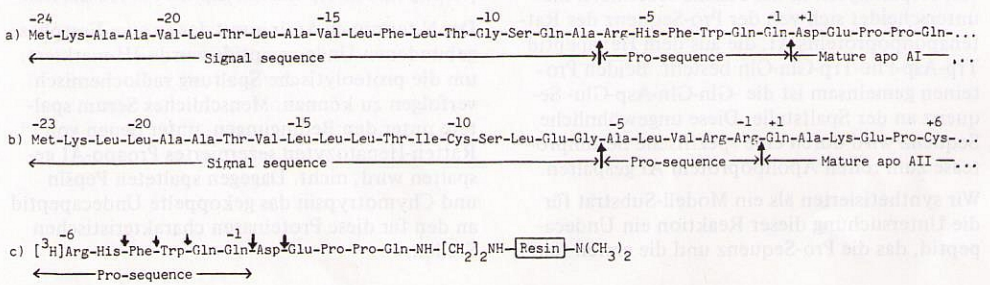

Fig. 1. Amino-acid sequence of the primary translation products of human liver apolipoprotein AI mRNA (a) and AII mRNA (b) and structure of the synthetic model substrate for pro-apo AI cleavage (c).

In Fig. 1c the arrows indicate the cleavage sites by pepsin (cf. Fig. 3).

and chymotrypsin degraded the resin-linked undecapeptide. The hydrolysis products were characterized by amino-acid hydrolysis of the peptides isolated by high-performance liquid chromatography.

Materials and Methods

Derivatized amino acids were purchased from Bachem (CH-4416 Bubendorf).

N^{α}-*t*-butoxycarbonyl-N^{im}-(,4-dinitrophenyl)histidin and N^{α},N^{ω}-bis(*t*-butoxycarbonyl)-[2,3-$^3H_2$]-L-arginine were synthesized and activated by esterification with 4-nitrophenol following established procedures[15,16]. All solvents were redistilled in an argon atmosphere. [2,3-$^3H_2$]-Arginine was a product of Amersham-Buchler, D-3300 Braunschweig. Glassware was silanized in 5% dimethyldichlorosilane in petroleum ether (30–60 °C). Symmetric butoxycarbonylamino-acid anhydrides (4 mmol) were prepared in dry methylene chloride (2 mol) with dicyclohexylcarbodiimide (2 mmol) in 1 m*l* methylene chloride.

The resin, a copolymer of *N,N*-dimethylacrylamide and 1,2-bis(acryloylamido)ethane functionalized with methyl *N*-acryloyl-*N*-methylglycinate, was reacted with ethylenediamine[17].

All protected amino acids were coupled as activated anhydrides, except the protected histidine and arginine, which were used as activated 4-nitrophenyl esters.

Each coupling cycle consisted of ten steps: 1) Five washings of the suspension with *t*-amylalcohol (30 m*l*/g resin, 2 min), 2) five washings with methylene chloride for 5 min and 3) five washings for 25 min., each in 40% trifluoroacetic acid, 2% mercaptoethanol in methylene chloride (30 m*l*/g resin), 4) five washings with methylene chloride, 5) five washings with *t*-amylalcohol, 6) ten washings with dimethylformamide, 7) three washings with 10% ethyldiisopropylamine in dimethylformamide for 2 min each (30 m*l*/g resin), 8) ten washings with dimethylformamide (25 m*l*/g resin, 2 min), 9) coupling of *t*-butoxycarbonyl-amino acid anhydride (6M excess) in dimethylformamide (20 m*l*/g resin, 20 min) and 10) five washings with dimethylformamide (2 min).

When 4-nitrophenyl ester-activated amino acids were coupled, a washing step with 6M excess of 1-hydroxybenzotriazol in dimethylformamide and three washings with dimethylformamide were introduced between steps eight and nine. The 6M excess of the nitrophenyl ester and 1-hydroxybenzotriazol in dimethylformamide was reacted for 4 h. Five final washings with dimethylformamide finished the cycle.

A semiautomated coupling machine was used[18].

t-Butoxycarbonyl and benzyl ester groups were cleaved with boron tris(trifluoroacetate)[19], the N^{im}-(2,4-dinitrophenyl) group with 2-mercaptoethanol at pH 8[16]. Amino-acid hydrolysis was performed in a Dow-Chemical Corp. analyser with *o*-phthalaldehyde as detecting reagent[20]. The resin-bound undecapeptide was sequenced by automated Edman degradation in a homemade solid phase sequenator, built according to Machleidt et al.[21,22]. The phenylthiohydantoin derivatives of the amino acids were separated by high-performance liquid chromatography, using mixed-bed nitrile and phenyl RP-columns and the linear gradient described before[23]. The high-performance liquid chromatography system consisted of two Altex-Beckman pumps, a BT 3030 detection unit (Biotronik) and the Hewlett-Packard integrator plotter model 3390 A.

Lipoprotein-deficient serum was obtained from freshly taken venous blood: Blood cells were sedimented at 2500 × *g* for 15 min. The clear supernatant was adjusted with solid potassium bromide to a density of 1.21 g/m*l* and centrifuged at 55000 rpm in a Beckman Ti 75 rotor for 20 h. The infranatant of the floating lipoprotein layer was recovered and dialysed against water, the pH adjusted with concentrated ammonia to 7.4 for 2 h.

Pepsin cleavage: 1 mg undecapeptidyl-resin was suspended in 1 m*l* 2% formic acid pH 1.9 and 70 μg pepsin was added (peptide: proteinase 50 : 1).

Chymotrypsin cleavage of 1 mg undecapeptidyl-resin was performed with 48 μg chymotrypsin in 1 m*l* 0.5M ammonium hydrogencarbonate buffer, pH 8.0.

The resin was sedimented by centrifugation and the supernatant lyophilized.

Peptides were separated on a RP 18 column (0.4 × 30 cm) with a gradient elution indicated in the Figures. Buffer A: 20mM sodium acetate pH 4; Buffer B: buffer A + acetonitrile (1 : 1); column temperature: 35 °C. Fractions were collected for counting of radioactivity.

Results

1) Synthesis of the resin-linked undecapeptide as model substrate

A polar resin, based on poly(*N,N*-dimethylacrylamide)[17,24] with methyl *N*-methylglycinate side chains was functionalized with ethylenediamine. The first amino acid glutamine was covalently linked by an amide bond to the derivatized resin. All amino acids except glutamine and arginine were activated with dicyclohexylcarbodiimide to their symmetric anhydrides, the latter were used as activated 4-nitrophenyl esters.

The activation in situ with dicyclohexylcarbodiimide was avoided because of the reduced activation rate in dimethylformamide, the most suitable solvent for this polar resin. The rate of aminolysis of the activated amino-acid esters was enhanced by excess 1-hydroxybenzotriazole[25]. The undecapeptide was labelled at the N-terminus with [2,3-$^3H_2$]arginine (Fig. 1 c); for synthesis, the amino and guanidino groups were protected.

The progress of the synthesis, carried out semiautomatically[18], was monitored after seven residues were linked to the resin. Aliquot portions of the intermediate hepta- and decapeptide-carrying resin were first deprotected and then completely hydrolysed. Acid-labile protecting groups such as benzyl ester groups and the *t*-

butoxycarbonyl group were released by the method of Pless and Bauer[19] who introduced boron tris(trifluoroacetate) in trifluoroacetic acid, a procedure which is equivalent to the hydrofluoride or hydrogen bromide in the acetic acid method[26,27] and avoids deterioration of tryptophane residues. The acidolysis is followed by the demasking of histidine with mercaptoethanol at pH 8 from the N^{im}-2,4-dinitrophenyl group[15]. The table lists the amino-acid stoichiometry of the hydrolysates of the polymer linked hepta- and decapeptide.

Table 1. Amino-acid analysis of the synthesized resin-linked hepta- and decapeptides after deprotection and hydrolysis.
The *o*-phthalaldehyde method does not register proline residues; the stoichiometry is based on aspartic acid.

	Heptapeptide		Decapeptide	
	theor.	found	theor.	found
Asp	1	1	1	1
Glu	4	5	4	5
Phe	–	–	1	1
His	–	–	1	0.83

Aspartic acid benzyl ester is more resistant to acid hydrolysis than glutamic acid benzyl ester, which might explain the high Glu/Asp ratio.

The sequential Edman degradation carried out in a solid phase automated sequenator[22] resulted in the expected structure of the decapeptide linked to the resin. The phenylthiohydantoin derivatives were identified by high-performance liquid chromatography on a reversed-phase mixed-bed nitrile-phenyl phase with a sodium acetate-acetonitrile gradient described before[23] (Fig. 2). From the integration of the peaks it is evident that each coupling step occurred with a yield of more than 99%.

The final coupling step of N^{α},N^{ω}-bis(*t*-butyloxycarbonyl)-[2,3-$^3H_2$]arginine (specif. act. 0.96 mCi/mmol) took place with a 95% yield as determined by the ^{3}H-radioactivity in the total hydrolysate of an aliquot portion of the resin-linked undecapeptide.

2) Attempts at enzymatic cleavage of resin-linked partial pro-apo AI sequence

a) Incubation with serum and lipoprotein-deficient serum

Samples of the resin-linked radiolabelled undecapeptide (0.1 μmol of peptide) were incubated for different time intervals with either whole serum or lipoprotein-deficient serum (d = 1.21 g/ml infranatant) and the radioactivity released into the medium was determined after sedimentation of the resin from the supernatant. The minute amounts of radioactivity released into the supernatant were the same as those determined in the control. Conditions for the incubations were identical with those which led to a 50 to 60% cleavage of rat pro-apo AI secreted from rat hepatocytes in suspension culture in the presence of rat serum or lipoprotein-deficient serum. Therefore the model particle is not suitable as substrate, although the correct N-terminal pro-sequence, the hexapeptide Arg-His--Phe-Trp-Gln-Gln linked to the first five amino acids of the mature form, protrudes from the hydrophilic resin.

b) Peptic and chymotryptic cleavage

The undecapeptide contains aromatic and acidic amino-acid residues which are prone to the proteolytic attack by pepsin and chymotrypsin. Incubations at pH 1.9 and pH 8.0, respectively, led to a release of 10% and 25% of [^{3}H]Arg-containing peptides within 90 min.

After incubation with pepsin the peptide mixture of the supernatant was separated by high-performance liquid chromatography in a sodium acetate/acetonitrile gradient on a RP-column (Fig. 3). Five bands of distinct retention times (t_R) were collected and the peptides were hydrolysed with acid for amino-acid analysis. Only two peptides, the hexapeptide Arg-His-Phe-Trp--Gln-Gln (t_R ≈ 36.69) and the dipeptide Arg-His (t_R = 12.31) were radioactive, the other peaks resembled Trp-Gln-Gln (t_R ≈ 17.70), Phe-Trp--Gln-Gln-Asp (t_R = 34.83) and free Trp (t_R = 10.06), indicating the cleavage sites marked by arrows in Fig. 1c.

The most effective cleavage rate by chymotrypsin could be explained by the smaller size of the enzyme, facilitating permeation into the pores of the resin loaded with the substrate peptide.

Discussion

Recent studies in our laboratory demonstrated that rat hepatocytes synthesize and process prepro-apolipoprotein AI to the intracellularly stable pro-apo AI form. Apolipoprotein AII is also synthesized primarily as a prepro-apo AII and released into the tubular system of the endoplasmic reticulum as pro-apo AII. Whereas pro-apo AI carries a hexapeptide with the unusual -Gln-Gln-Asp-Glu-cleavage site, the pro-pentapeptide of apo AII ends in the two basic residues Arg-Arg, common to most proproteins[28]. Obviously pro-apo AI cannot be further processed in the cell and is secreted as such, pro-apo AII however is processed to a large extent, to mature apo AII, but pro-apo AII is also secreted.

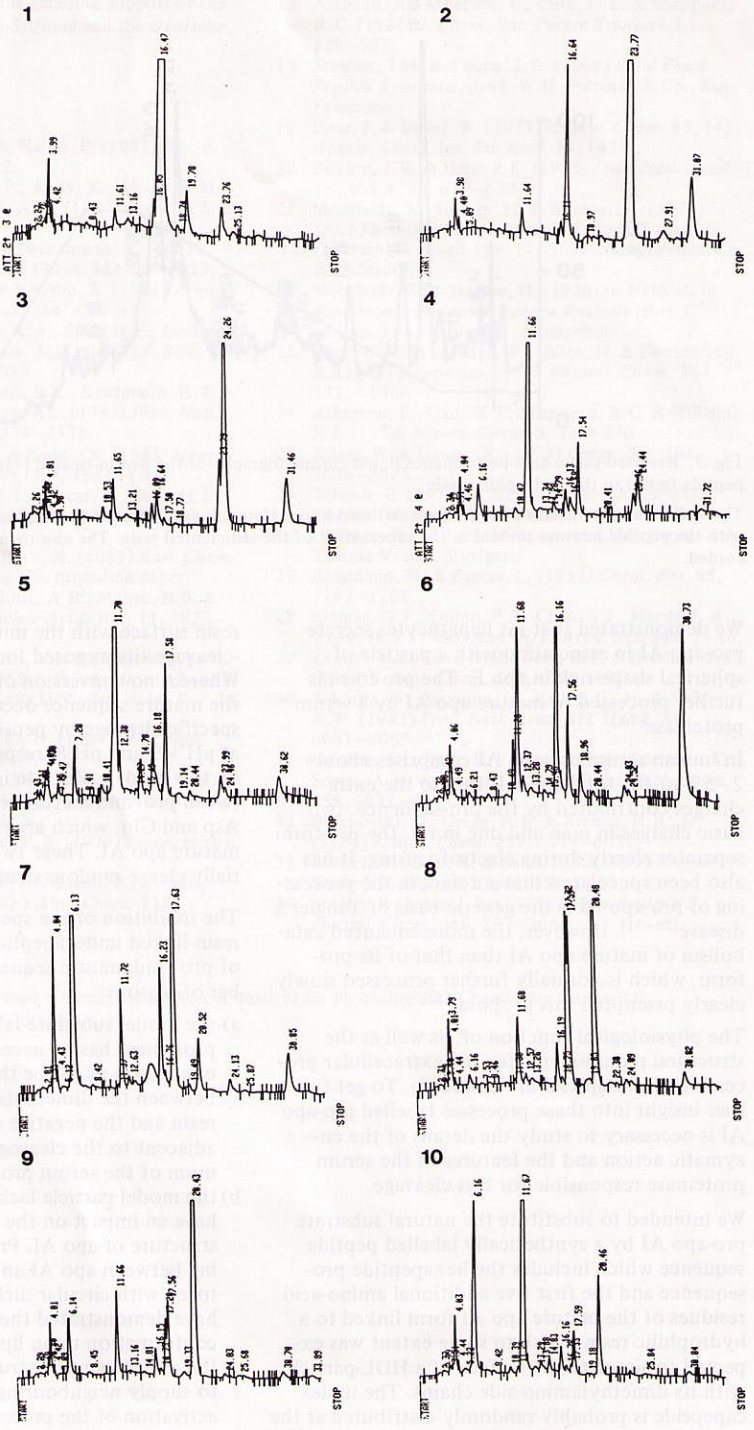

Fig. 2. High-performance liquid chromatography of the phenylthiohydantoin derivatives of amino acids released from the resin-linked decapeptide by sequential Edman degradation in a solid phase automated sequenator[22].

The conditions of the chromatographic separation are described under ref.[3].

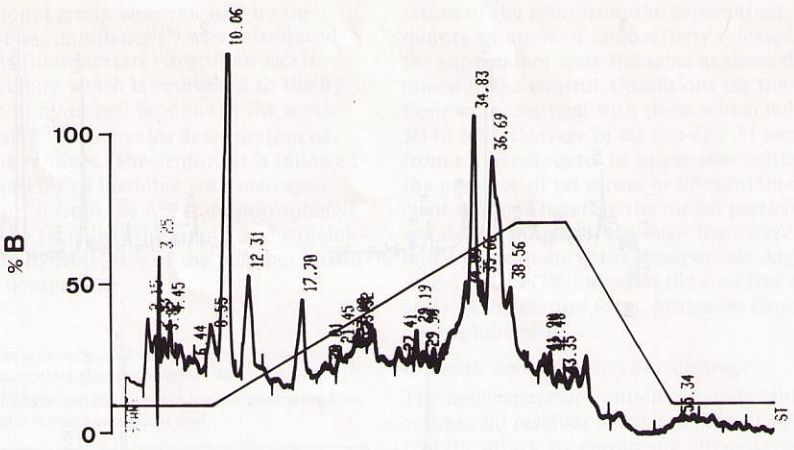

Fig. 3. Reversed-phase high-performance liquid chromatography of the pepsin-treated [^{3}H]arginine-labelled undecapeptide linked to the hydrophilic resin.

The sodium acetate/acetonitrile gradient outlined under Materials and Methods was applied to a RP 18 column loaded with the peptide mixture present in the supernatant of the sedimented resin. The absorbance at 210 nm was recorded.

We demonstrated that rat hepatocytes secrete pro-apo AI in association with a particle of spherical shape rich in apo E. The pro-form is further processed to mature apo AI by a serum proteinase[7,8].

In human serum pro-apo AI comprises about 2–5% of the total apo AI. Due to the extra charges contributed by the pro-sequence, two basic charges in man and one in rat, the pro-form separates clearly during electrofocusing. It has also been speculated that a defect in the processing of pro-apo AI is the genetic basis of Tangier's disease[29–31]. However, the more enhanced catabolism of mature apo AI than that of its pro-form, which is normally further processed slowly, clearly prompted this proposal[32].

The physiological function of, as well as the structural requirements for the extracellular processing of pro-apo AI are unknown. To get further insight into these processes labelled pro-apo AI is necessary to study the details of the enzymatic action and the features of the serum proteinase responsible for this cleavage.

We intended to substitute the natural substrate pro-apo AI by a synthetically labelled peptide sequence which includes the hexapeptide pro-sequence and the first five additional amino-acid residues of the mature apo AI form linked to a hydrophilic resin which to some extent was expected to simulate the surface of a HDL-particle with its dimethylamino side chains. The undecapeptide is probably randomly distributed at the resin surface with the unusual -Gln-Gln-Asp-Glu--cleavage site exposed for the proteolytic attack. Whereas no conversion of the pro-sequence to the mature sequence occurred, the sites for the specific cleavage by pepsin and chymotrypsin at pH 1.9 and pH 8, respectively, were exposed on this model substrate including cleavage between pro- and mature sequence and between Asp and Glu, which are residues 1 and 2 of mature apo AI. These two proteinases preferentially cleave random structures of polypeptides.

The inhibition of the specific cleavage of the resin-linked undecapeptide at the joining point of pro- and mature sequence might have a number of reasons:

a) the model substrate is unsuitable because the proteinase has no access to the cleavage site; one reason might be the ionic interactions between the dimethylamino groups of the resin and the negative charges of Asp and Glu, adjacent to the cleavage site at the pH optimum of the serum proteinase;
b) the model particle lacks lipids which might have an impact on the secondary and tertiary structure of apo AI. Previous studies on binding between apo AI and phospholipids monitored with circular dichroism measurements have demonstrated the dramatic changes in conformation upon lipid binding[33,34];
c) the undecapeptide structure might be unable to supply neighbouring group interaction for activation of the proteolytic attack.

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