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# Human Aminopeptidases: A Review of the Literature

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**Summary:** The aminopeptidases constitute a group of enzymes with closely related activities. In clinical chemistry the analysis of the aminopeptidases and of their multiple forms in serum has for a long time been hindered by considerable confusion concerning their identification, and by a lack of characterization. This is in part due to the often large, and sometimes overlapping substrate specificities of the aminopeptidases.

This paper reviews the biochemical properties of the different aminopeptidases, the specificities of the assays used for their analysis in serum, some aspects of their multiple forms — which are especially known to occur for alanine aminopeptidase (EC 3.4.11.2) — and the importance of the determination of aminopeptidases and their multiple forms in clinical chemistry.

## Introduction

Aminopeptidases are enzymes which hydrolyse peptide bonds near the N-terminal end of polypeptides. They can be subdivided into aminopeptidases which hydrolyse the first peptide bond (aminoacyl-peptide hydrolases and iminoacyl-peptide hydrolases) and those which remove dipeptides from polypeptide chains (dipeptidyl-peptide hydrolases). Some peptidases act only on dipeptides or tripeptides and may also be considered as aminopeptidases.

Aminopeptidases are present in many human tissues and body fluids. They are generally zinc-metalloenzymes. They are thought to be involved in the metabolism of proteins and various peptide hormones.

According to the method and conditions of analysis, several forms of aminopeptidase activity can be determined in human plasma and other tissues. This heterogeneity has several causes:

— different gene loci; several proteins are synthesized with an aminopeptidase activity. These enzymes differ in immunological properties, substrate specificities, pH optima, activators, etc. A list of some human aminopeptidases acting on polypeptides is given in table 1.

— substrate specificities; the different aminopeptidases have a closely related enzymatic activity with sometimes broad specificities. These specificities overlap, so that many natural and synthetic substrates may be hydrolyzed by more than one enzyme. This is especially the case when the substrate is a polypeptide.

— post-translational modifications; these are especially known to occur for alanine aminopeptidase. The modifications which lead to the presence of multiple forms are:
  - glycation
  - limited proteolysis
  - aggregation with other proteins or phospholipids.

Taking some of these aspects into consideration, we shall discuss the main human aminopeptidases of interest in clinical chemistry, excluding dipeptidases and dipeptidyl-peptide hydrolases.

Tab. 1. Human aminopeptidases

| Enzyme | Substrate specificities | Remarks |
|---|---|---|
| Leucine aminopeptidase EC 3.4.11.1 | leu-X- (AA-X-)* | $Mg^{2+}$, $Mn^{2+}$ activation; basic pH optimum; does not hydrolyse chromogenic substrates |
| Alanine aminopeptidase EC 3.4.11.2 | ala-X- (AA-X-) | some activation by $Co^{2+}$ |
| Cystyl aminopeptidase EC 3.4.11.3 | leu-X- (cys-X-, AA-X-) | no inhibition by bestatin and amastatin; heat labile |
| Aminopeptidase A EC 3.4.11.7 | asp-X- glu-X- | activated by $Ca^{2+}$; for glu-substrates also activated by $Ba^{2+}$ |
| Aminopeptidase B EC 3.4.11.6 | lys-X- arg-X- | activation by $Cl^-$ and $Br^-$; unstable |
| Aminopeptidase P EC 3.4.11.9 | X-pro- | $Mn^{2+}$ activation |
| Tripeptidase EC 3.4.11.4 | leu-(gly-gly) gly(gly-gly) | acts on tripeptides only; no inhibition by amastatin |
| Dipeptidases EC 3.4.13.- | AA-AA | act on dipeptides only |
| Dipeptidyl-peptidases EC 3.4.14.- | (AA-AA)-X- | remove dipeptides |

*AA = amino acid

## Aminopeptidases from Different Gene Loci

### Leucine aminopeptidase

Leucine aminopeptidase (α-aminoacyl-peptide hydrolase (cytosol), EC 3.4.11.1) was discovered by *Linderstrøm-Lang* (1) in 1929. It hydrolyses preferentially, but not exclusively, peptide bonds adjacent to an N-terminal leucine residue, as in leucinamide and leucylglycine (2, 3). Leucine aminopeptidase has mainly been studied in bovine lens and pig liver (4–7). It is a zinc-metalloenzyme, generally localized in cytosolic subfractions, and it is present in liver, lung, stomach, kidney, intestine, serum and leukocytes, as well as other tissues (3, 8, 9).

Leucine aminopeptidase is immunologically distinct from alanine aminopeptidase (8). *Ledeme* et al. (10) purified two forms of leucine aminopeptidase activity from human liver which differed in their isoelectric points. *Kohno* et al. (3) recently purified the leucine aminopeptidase from liver cytosol by immunoaffinity chromatography. Liver leucine aminopeptidase is a hexamer ($M_r$ 360 000) consisting of three dimers with two different subunits each ($M_r$ 53 000 and 65 000). Human leucine aminopeptidase has an optimum at pH 10, is typically activated by $Mg^{2+}$ and $Mn^{2+}$, and inhibited by $Zn^{2+}$, $Co^{2+}$, complexing agents, bestatin and amastatin. Human liver leucine aminopeptidase was not found to hydrolyse the chromogenic substrates leucyl-4-nitroanilide, leucyl-β-naphthylamide or alanyl-4-nitroanilide (3, 10).

### Alanine aminopeptidase

Alanine aminopeptidase (α-aminoacyl-peptide hydrolase (microsomal), EC 3.4.11.2, arylamidase, aminopeptidase M, aminopeptidase N) is probably the aminopeptidase which has been studied most extensively. Alanine aminopeptidase hydrolyses preferentially natural or synthetic substrates with an N-terminal alanine residue. Other amino acids, especially leucine, may also be removed hydrolytically, with the exception of proline. The lowest $K_m$ values are found with methionine-substrates (2, 11–16).

Alanine aminopeptidase may hydrolyse several biologically active peptides, e. g. met-lys-bradykinin and lys-bradykinin (17). Alanine aminopeptidase from rat brain and hog aorta are capable of hydrolysing ($met^5$)-enkephalin and ($leu^5$)-enkephalin (18–20). In enzyme assays the most frequently used substrates are 4-nitroanilides and β-naphthylamides of alanine and leucine.

Human alanine aminopeptidase has been found to be present in virtually all tissues studied, with relatively high specific activities in the brush border membranes

of kidney proximal tubules and intestine and in bile canalicular membranes (21 – 27). Human alanine aminopeptidase has been purified from liver (8, 12, 28 – 30), kidney (11, 31 – 33), intestine (13, 34, 35), placenta (31, 36) and blood plasma (37, 38). Relative molecular mass estimations of the enzyme range from about 150 000 (39 – 41) (obtained by electrophoresis of the non-purified serum enzyme) to about 240 000 for purified enzymes by gel filtration (16, 28, 42). *Starnes & Behal* (28) noticed an equilibrium between a 118 000 monomeric form and a 235 000 dimeric form of liver alanine aminopeptidase in dilute salt solutions. Each 118 000 unit contains one atom of zinc (43). Alanine aminopeptidase is a glycoprotein, with a carbohydrate part presenting between 12 and 21% of its mass, depending on the tissue source (11, 28, 31, 37). Alanine aminopeptidase may contain a large number of sialic acid groups, which explains the rather low isoelectric points, from pH 3.3 – 3.6 (liver, serum enzyme) to pH 4.7 (kidney, pancreas) (8, 28, 40, 44).

Alanine aminopeptidase is generally found in membrane fractions obtained by ultracentrifugation. It can be solubilized from tissues by autolysis or by proteolytic enzymes such as papain, bromelain and trypsin, or by extraction with detergents (22, 25). Studies on alanine aminopeptidase of pig intestine have shown that alanine aminopeptidase is anchored in the membrane by a small hydrophobic polypeptide which is removed by the solubilizing enzymes (45, 46).

Alanine aminopeptidase is activated by $Co^{2+}$. Some other metal ions, complexing agents and amino acids are inhibitors (13, 33, 37, 43, 47). A very potent inhibitor is amastatin and, to a lesser degree, bestatin and puromycin (15, 37). The pH optimum of alanine aminopeptidase is buffer- and substrate-dependent, generally between pH 6.5 – 8.5 (48 – 50).

## Cystyl aminopeptidase

Cystyl aminopeptidase (α-aminoacyl-peptide hydrolase, EC 3.4.11.3, oxytocinase) can be detected in serum and amniotic fluid during pregnancy (51 – 53). The physiological substrate of cystyl aminopeptidase appears to be oxytocin, a peptide with an N-terminal cystine, and which induces uterine contraction. Cystyl aminopeptidase is mainly localized in placental lysosomes (54).

*Lalu* et al. (55) compared the biochemical characteristics of cystyl aminopeptidase preparations purified from placenta and maternal serum. The relative molecular mass of both enzymes was 320 000, which is the same as found by *Oya* et al. (39) for serum cystyl aminopeptidase. The most rapidly hydrolysed substrate was leucyl-4-nitroanilide, followed by leucyl-β-naphthylamide and S-benzyl-cysteyl-4-nitroanilide. The preparations also showed lysyl-β-naphthylamidase activity, while the $V_{max}$ of cystyl-di-β-naphthylamide hydrolysis was only 7% of that of leucyl-β-naphthylamide. One difference between the two purified preparations was the relatively high alanyl-β-naphthylamidase acitivity of the placental enzyme preparation. The preferential hydrolysis of leucyl-arylamides by cystyl aminopeptidase is a general finding (36, 38, 40, 48, 56). Some characteristic properties of cystyl aminopeptidase which distinguish it from some other aminopeptidases, are its heat lability and resistance to methionine, bestatin and amastatin inhibition (15, 57).

It is generally accepted that cystyl aminopeptidase in serum is derived from placental lysosomes (54, 55, 58), but comparisons of studies on placental and serum cystyl aminopeptidase are hindered by the fact that the placenta contains several other soluble and membrane-bound aminopeptidases (59, 60). The substrate specificities are rather broad, and pH optima and the action of effectors are often found to be substrate- or tissue-dependent (55, 61, 62). It has been shown that the placenta also contains aminopeptidase A (63) and microsomal alanine aminopeptidase, which is immunologically different from serum cystyl aminopeptidase but identical with kidney alanine aminopeptidase (31, 36). Several groups have observed a placental enzyme which shares with cystyl aminopeptidase its insensitivity to methionine, its heat lability and the capacity to hydrolyse cystyl-di-β-naphthylamide, while it has in common with alanine aminopeptidase its microsomal localization and electrophoretic mobility (52, 54, 58). It is also present in meconium-contaminated amniotic fluid and colostrum (52, 64). A possible explanation for these findings would be some hydrolysis of cystine substrates by microsomal alanine aminopeptidase (65), but the enzyme activity was not found in normal or pregnancy serum.

It is clear that more immunological studies are needed to identify further the various aminopeptidase activities in the placenta and in pregnancy serum.

## Other aminopeptidases

Some other aminopeptidases have been detected in humans, sometimes in serum, but for most of these the clinical interest has hardly been explored. They are summarized beneath, since they may gain interest and are possible sources of interference in the various enzyme assays.

*Tripeptide aminopeptidase*

Tripeptide aminopeptidase (α-aminoacyl-dipeptide hydrolase, EC 3.4.11.4) only acts on the N-terminal peptide bond of tripeptides. Few studies have been conducted on the human enzyme. *Kanda* et al. (66) found the enzyme to be widely distributed in cytosolic subfractions of human tissues such as liver, intestine, kidney, brain and lymphocytes. Usual substrates for tripeptide aminopeptidase are leucyl-glycyl-glycine and triglycine. Tripeptidases from animal tissues generally have a relative molecular mass between 50 000 and 70 000, with a neutral pH optimum, and are inhibited by bestatin (67). Amastatin is not an inhibitor; this property has been used to distinguish tripeptide aminopeptidase activity in serum from other aminopeptidases (68).

*Aminopeptidase A*

Aminopeptidase A (*L*-α-aspartyl (L-α-glutamyl)-peptide hydrolase, EC 3.4.11.7, angiotensinase A) specifically hydrolyses N-terminal aspartyl or glutamyl residues from peptides or synthetic substrates (69). A biological substrate is angiotensin, which possesses an N-terminal Asp residue. This enzyme might therefore also be called aspartyl aminopeptidase. The enzyme has been purified from human placenta and pregnancy serum. It is present in various reproductive organs (63, 70, 71). The relative molecular mass of the human serum enzyme has been estimated as 260 000. Aspartyl aminopeptidase is characteristically activated by $Ca^{++}$ with the substrates glu-β-naphthylamide and asp-β-naphthylamide. Hydrolysis of glu-β-naphthylamide but not of asp-β-naphthylamide is also activated by $Ba^{++}$ ions. Activity towards leu-β-naphthylamide is absent and is very low towards ala-β-naphthylamide (70).

*Aminopeptidase P*

Aminopeptidase P (EC 3.4.11.9) occupies a special place in this group of aminopeptidases, since it is able to split N-terminal peptide bonds with a secondary proline residue (72). Natural substrates are possibly bradykinin and substance P (73). The enzyme has been purified from human lung (74). The relative molecular mass was estimated as 188 000. The study of *Appel* (75) on human serum proline arylamidase seems to refer to a dipeptidase, proline aminopeptidase (EC 3.4.13.8).

*Aminopeptidase B*

Aminopeptidase B (EC 3.4.11.6) shows a specificity for N-terminal lysyl and arginyl amino acids. It has been purified from the cytosolic fraction of human liver and skeletal muscle (12, 76). The relative molecular mass of human aminopeptidase B was estimated as 72 000. It is an unstable, thiol-dependent enzyme. It hydrolyses preferentially, but not exclusively, dipeptides. Aminopeptidase B acts on synthetic lysyl- or arginyl-substrates, but not on ala-β-naphthylamide or leu-β-naphthylamide. The enzyme shows a remarkable activation by $Cl^-$ or $Br^-$ ions (12, 76). It is inhibited by chelating agents and bestatin (76, 77).

## Substrates and Methods of Analysis of Aminopeptidases from Serum

### Leucine aminopeptidase

As mentioned above, leucine aminopeptidase does not hydrolyse the chromogenic substrates leucyl-4-nitroanilide and leucyl-β-naphthylamide (3, 10, 78). Leucine aminopeptidase activity is most often measured with the substrate leucinamide, which it hydrolyses to leucine and free ammonia (79). For the assay of purified enzyme preparations, the decrease in absorbance may be measured directly at 238 nm (80). Several groups have proposed methods in which the liberated $NH_3$ is detected. *Plaquet* et al. (81) and *Tamura* et al. (82) used the reaction of *Berthelot* with nitroprusside for the determination of leucine aminopeptidase in human serum and rat plasma, respectively. Continuous monitoring assays have been described by *Hafkenscheid & Kohler* (83) and by *Kanno* et al. (84). In their methods the liberated ammonia reacts with 2-oxoglutarate in the presence of glutamate dehydrogenase. The decrease in NADPH concentration, which is used in the reaction, is measured at 340 nm.

*Takamiya* et al. (85) proposed a method in which the leucine generated by the hydrolysis of leucinamide or leucyl peptides is reduced by the enzyme leucine dehydrogenase. The NADH formed in the reaction is measured at 340 nm.

Despite the frequent use of leucinamide as substrate for leucine aminopeptidase, it seems not to be hydrolysed specifically by this enzyme only (50). *Plaquet* et al. (81) found two forms of leucinamidase activity in serum with different pH optima. The enzyme with a neutral pH optimum was inhibited by $Mg^{2+}$ and was predominant in normal sera. Similarly, *Kanno* et al. (84) showed by electrophoresis that there are two leucinamidase bands in human serum. One of the two forms also hydrolysed leucyl-4-nitroanilide and was thus an arylamidase, propably alanine aminopeptidase.

*Plaquet* et al. (81) eliminated the neutral activity by performing the reaction at pH 9 in the presence of $Mg^{2+}$ ions, which activates leucine aminopeptidase and inhibits alanine aminopeptidase.

Dependence of substrate specificity on pH was also found by *Haschen* et al. (86) in an assay with leucyl-hydrazide as substrate and *p*-dimethylaminobenzaldehyde as staining agent, and by *Fleisher* et al. (87) with leucyl-glycine as substrate. Like leucinamide, these substrates were also susceptible to hydrolysis by alanine aminopeptidase at neutral or weakly basic pH, and the assays were performed at pH 10.

Although the methods with an enzymatic indicator reaction have the advantage that they may be used in an automated continuous assay, they have to be performed at a lower pH (8.2—8.3). Alanine aminopeptidase is still active at this pH, which may explain why a good correlation between leucinamide and leucyl-4-nitroanilide hydrolysing activities has been observed for some groups of samples (83, 85). Interference by alanine aminopeptidase may also explain the difference in upper reference limits which has been observed with the methods at basic pH [12 U/l, (81) and 1.6 U/l (86)] and a continuous method [65 U/l, (83)].

Other substrates, which have been used for the detection of leucine aminopeptidase, are leucyl-glutamic acid by *Kusukabi* (88) with an indicator reaction on the liberated glutamate, leucylglycine with a ninhydrin reaction by *Fleisher* et al. (89), and the synthetic compound phe-3-thia-phe by *Hwang* et al. (90). After enzymatic hydrolysis of this substrate, the thiol group is released and stained with *Ellman*'s reagent. The authors found a 20-fold activity of leucine aminopeptidase as compared to alanine aminopeptidase for this substrate, but the enzyme sources were not stated. A disadvantage was some inhibition of leucine aminopeptidase by *Ellman*'s reagent.

## Alanine aminopeptidase

The synthetic substrate leucyl-β-naphthylamide was used for the first time thirty years ago by *Goldberg & Rutenburg* (91, 92) in what was thought to be the analysis of leucine aminopeptidase. Later it became clear that this substrate and leucyl-4-nitroanilide, which was introduced in 1962 by *Tuppy* et al. (93), were not hydrolysed by leucine aminopeptidase, but by another enzyme (78, 87, 94), which furthermore hydrolysed alanyl-substrates faster than leucyl-substrates (48, 94, 95). The name alanine aminopeptidase was proposed 20 years ago for this enzyme by *Rehfeld* et al. (96). However, the use of leucyl-substrates for the detection of alanine aminopeptidase has continued along with the designation of alanine aminopeptidase as leucine aminopeptidase, leucine arylamidase or leucyl-β-naphthylamidase, which has led to a certain confusion.

The most frequently used method with leucyl-4-nitroanilide as substrate appears to be that of *Nagel* et al. (97). An optimized assay has been recommended by the German Society for Clinical Chemistry (DGKC) (98). This method with a high substrate concentration was further modified by *Hafkenscheid & Dijt* (99).

The liberated nitroaniline is strongly coloured, which makes continuous monitoring possible. The product of the hydrolysis of leucyl-β-naphthylamide, β-naphthylamine, is only weakly coloured. It may be measured by fluorimetry, but generally a coupling reaction with a diazonium salt is used.

A drawback of the use of leucyl substrates is the fact that they are the preferential substrates of cystyl aminopeptidase, which hydrolyses them even better than synthetic cysteine substrates, and that their use contributes to the confusion with leucine aminopeptidase.

*Tokioka-Terao* et al. (100) developed a radioimmunoassay of human serum alanine aminopeptidase. They found alanine aminopeptidase protein concentrations to correlate well with activity towards alanyl-β-naphthylamide in normal samples and in cases of hepatobiliary disease. However, they found some increase in enzymatic activity with this substrate — and still much more with leucyl-4-nitroanilide — in pregnancy sera, while alanine aminopeptidase protein concentrations were the same as in non-pregnant women. Hence, the increases measured with these substrates are due to cystyl aminopeptidase in serum. Yet, no increase in alanyl-4-nitroanilide hydrolysing activity has been noticed in pregnancy sera (48, 101). This would indicate a better specificity of alanyl-4-nitroanilide than of alanyl-β-naphthylamide, but this remains to be confirmed.

There is now a certain agreement on the use of alanyl-4-nitroanilide as substrate for determination of alanine aminopeptidase activity in serum and urine (48, 49, 101—105). It has the advantage of specificity, it has good solubility, and because of the high molar absorbance of 4-nitroaniline it may be used in a continuous assay, which makes it suitable for automatic analysers. Alanyl-β-naphthylamide is more convenient for histochemical analysis and for enzyme detection on electrophoretograms, because the product diffuses only slowly and the diazo-coupled product precipitates; this makes long incubation times possible (92, 106, 107).

Other substrates which have been proposed for alanine aminopeptidase determination are leucyl-3-carboxy-4-hydroxyanilide in an endpoint method (108), and fluorogenic substrates (109, 110).

### Cystyl aminopeptidase

Several synthetic substrates have been employed in the analysis of serum cystyl aminopeptidase; these are generally di-substituted derivates of cystine, e. g. cystyl-di-β-naphthylamide, S-benzyl-cysteyl-β-naphthylamide, cystyl-bis-4-nitroanilide and S-benzyl-cysteyl-4-nitroanilide (93, 111–114). The relative activity towards these different substrates, the pH optimum and the influence of effectors depend on the tissue source, which indicates lack of substrate specificities (61, 62). *Uete* et al. (62) concluded that S-benzyl-cysteyl-4-nitroanilide was the most specific of four cystyl aminopeptidase substrates tested. S-benzyl-cysteyl-4-nitroanilide has effectively gained preference (115), because of its relatively good solubility, the fact that among these cystine substrates it is hydrolysed the most rapidly by cystyl aminopeptidase and that it is easy to use in an automated assay (116–118).

*Mizutani* et al. (119) measured cystyl aminopeptidase activity in serum with leucyl-β-naphthylamide as substrate. The hydrolysis of the same substrate by alanine aminopeptidase was inhibited by methionine. The activity measured in this way was found to correlate well with hydrolysis of cystyl-di-β-naphthylamide. A fluorimetric method with S-benzyl-cys-methylcoumarin was proposed by *Suzuki* et al. (120).

## Multiple Forms of Alanine Aminopeptidase

Among the various aminopeptidases, the heterogeneity of alanine aminopeptidase has been most widely studied. Electrophoretic, chromatographic and immunological techniques have been used for this purpose. A first review on this subject was published in 1970 by *Boivin* et al. (23).

Several groups have compared alanine aminopeptidase from different human tissues by immunological techniques, but no differences in antigenicity have been found (121–123). It thus appears that all alanine aminopeptidase isoforms originate from a single gene product and that the heterogeneity is due to post-translational modifications. Three mechanisms causing the heterogeneity have been identified: glycation, limited proteolysis and aggregation with other molecules.

### Glycation

Alanine aminopeptidases from various tissues differ in electrophoretic mobility, heat sensitivity and susceptibility to lectin precipitation (23, 24, 42, 96, 124, 125). Treatment with neuraminidase abolishes the differences in electrophoretic mobility of the anodic bands of alanine aminopeptidase from various tissues (24). *Böhme* et al. (44) compared the carbohydrate groups of alanine aminopeptidases from liver, kidney and pancreas. They found the liver enzyme to contain the highest number of sialic acid groups, and further that sialic acid contents correlate well with molecular charges. These therefore appear to be the main cause of intertissular variation of alanine aminopeptidase on electrophoresis gels.

*Böhme* et al. (44) also found a variation in the number of glucose groups. *Lorentz* (125) found that differences in precipitation of alanine aminopeptidase from different tissues by various lectins persist after neuraminidase treatment, which also indicates variations in sugar groups other than sialic acids.

*Intra*tissular variations in molecular charge have been found for the pancreas enzyme and the kidney enzyme (14, 44). Recently it was shown that normal human serum contains two isoforms of alanine aminopeptidase, which differ in sialic acid content (40).

Alanine aminopeptidases from cancerous tissues differ from alanine aminopeptidases from the corresponding normal tissues in electrophoretic mobility and behaviour on ion-exchange media (9, 26, 126, 127). These differences are also attributed to variations in sialic acid content, and no immunological differences have been established (9, 122).

### Multimolecular complexes

Liver alanine aminopeptidase in its native form is an amphiphilic protein, anchored to the outer side of canalicular plasma membranes (22, 127, 128, 129). Enzymes with a similar localization are γ-glutamyltransferase (EC 2.3.2.2) and alkaline phosphatase (EC 3.1.3.1). For all these enzymes slower- or non-migrating bands may be detected upon polyacrylamide or agarose gel electrophoresis of sera from patients with liver disease (83, 106, 107, 124, 130–132). Similarly, enzyme forms of large size may be found in these sera by gel filtrations (133–135).

In 1972, *Shinkai & Akedo* (134) noticed a resemblance in the composition of the high molecular-mass forms and the liver plasma membrane, and they concluded that the large forms represented multienzyme complexes derived from the liver plasma membrane. *De*

*Broe* et al. (136) isolated vesicles from sera of patients with cholestasis, which contained the membranous enzymes alanine aminopeptidase, alkaline phosphatase, γ-glutamyltransferase and 5'-nucleotidase, but no cytosolic enzymes. The vesicles were thought to be plasma membrane fragments. They subsequently established that the 'shedding" of plasma membrane fragments into the surrounding medium is a common phenomenon in living cells (137). The plasma membrane fragments in human serum appear to be derived from liver cells and not to be of biliary origin (129). Similar fragments in urine originate from the brush border membrane of the kidney proximal tubuli (138, 139).

Amphiphilic forms of alanine aminopeptidase, alkaline phosphatase and γ-glutamyltransferase may be obtained in vitro by incubation of plasma membranes with detergents or bile salts (45, 140–143). The enzymes appear in a high molecular mass form, which is due to an attachment of the hydrophobic domain to other molecules (143, 144). Since amphiphilic, aggregated forms of alanine aminopeptidase, alkaline phosphatase and γ-glutamyltransferase are also present in sera from patients with cholestasis, it has been supposed that these forms may be solubilized in vivo by the action of bile salts on the liver plasma membrane (145, 146). Incubation of serum or urine with Triton X-100 dissociates the aggregates; complexes of enzymes with detergent molecules which bind to the hydrophobic domain are obtained (107, 147–149).

The composition of the multimolecular complexes of alanine aminopeptidase, γ-glutamyltransferase and alkaline phosphatase has been analysed by electrophoresis, gel filtration and precipitation techniques. *Crofton & Smith* (131) found the three enzymes to be associated with lipoprotein X. Aggregated forms of alanine aminopeptidase of intermediate relative molecular mass (400–600 000) and high $M_r$ (< 1 000 000) were found by *Wenham* et al. (135) upon gel filtration of cholestatic sera. *Sanderink* et al. (40) found several groups of multimolecular forms by the use of micro-two-dimensional electrophoresis (fig. 1). Some alanine aminopeptidase forms of intermediate $M_r$ may be precipitated by antibodies against apolipoprotein A or by polyanions, which indicates the existence of high density lipoprotein (HDL-) alanine aminopeptidase complexes (135, 150). Complexes of γ-glutamyltransferase and HDL have also been demonstrated (135, 151, 153), but not of alkaline phosphatase and HDL (135).

Alanine aminopeptidase, alkaline phosphatase and γ-glutamyltransferase may also be associated with apolipoprotein B or low density lipoproteins (131, 150, 152, 154).

While the composition of the multimolecular complexes is becoming better understood, their origin remains partly abscure. *Wenham* et al. (135, 144) observed that amphiphilic enzyme forms, which were solubilized from liver tissue by bile salts, reaggregated when the bile salt concentration was lowered. Moreover, they were not able to extract intermediate molecular mass complexes of γ-glutamyltransferase from liver tissue in vitro, unless in the presence of serum (143). These results would indicate that the multimolecular complexes may be formed by reaggregation of amphiphilic proteins in serum.

On the other hand, one would expect such a mechanism to result in a random distribution of the various complexes. However, the micro-2-dimensional electrophoresis patterns of the three enzymes (alanine aminopeptidase, alkaline phosphatase, γ-glutamyltransferase) from similar sera are clearly different (150) and so are the elution profiles of the three enzymes on a gel filtration column and the net charges of the complexes (135, 148). Differences in lipoprotein composition have also been established by immunoprecipitation studies. These results would be more consistent with a specific composition of the enzyme-lipoprotein complexes, reflecting their origin. At this moment neither of the two possibilities can be completely excluded.

Complexes of numerous serum enzymes with immunoglobulins have been detected. The specificity of the enzyme-immunoglobulin bond has been established in some cases. Complexes of alanine aminopeptidase with IgA and light (λ-) chains were found by *Sudo & Kanno* (155). The same group observed an alanine aminopeptidase-IgG(κ) complex in the serum of a patient with rheumatoid arthritis, together with complexes of immunoglobulins with other enzymes (156).

## Limited proteolysis

Autolysis of liver tissue or incubation of membrane fractions with proteolytic enzymes such as papain, trypsin and bromelain result in the solubilization of alanine aminopeptidase (21, 22, 25, 45, 140). The hydrophobic, anchoring part of the enzyme is cleaved off, so that the solubilized enzyme has a hydrophilic character (45). Although most studies on this subject have been performed on animal tissues, the model also seems to be true for human alanine aminopeptidase.

The amphiphilic, aggregated form of alanine aminopeptidase in serum can be dissociated from the multimolecular complexes by incubation with proteolytic enzymes in the same way that it is separated from

membranes. This also results in the appearance of low molecular mass, hydrophilic forms (40, 107, 131, 144).

Alanine aminopeptidases in normal serum have also a hydrophilic character. These forms are thought to be derived from the liver. In liver disease, alcohol consumption or drug intake, additional hydrophilic forms, named F-alanine aminopeptidases because of their *fast* mobility in polyacrylamide gel electrophoresis, may be detected in serum (40, 41). These forms have a slightly lower molecular mass than the normal alanine aminopeptidases (fig. 1). Similar forms may be obtained by incubation of normal serum alanine aminopeptidases or multimolecular complexes with papain. These additional forms are thus probably cleaved at a site different from that under normal conditions. The cause of this different proteolysis has not been elucidated, but it may be related to the induction of membrane proteases or perturbances of the liver plasma membranes (40, 41).

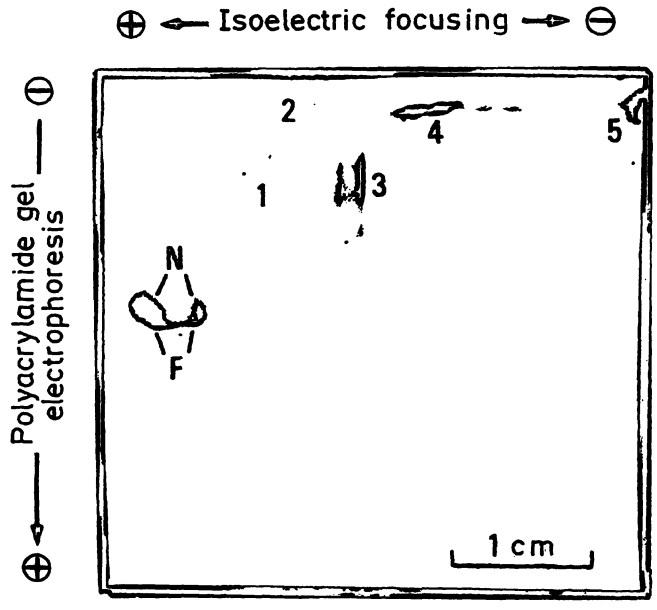


Fig. 1. Micro-two-dimensional electrophoresis gel of serum stained for alanine aminopeptidase (AAP). N: normal forms. F: F (fast)-AAP. 1 – 5: multimolecular complexes (with modifications from l.c. (40)).

### Multiple forms of other aminopeptidases

As in the case of alanine aminopeptidase, no evidence of genetic polymorphism has been reported for leucine aminopeptidase or cystyl aminopeptidase.

Two forms of leucine aminopeptidase were observed by *Ledeme* et al. (10) in human liver which could be separated by DEAE-Sephacel chromatography. A leucine aminopeptidase isoform has been observed by *Maekawa* et al. (156) in the serum of a patient with rheumatoid arthritis. This isoform was a leucine aminopeptidase-IgG complex.

As mentioned above, a heterogeneity observed for cystyl aminopeptidase may often be related to a lack of specificity of substrates. However, two isoforms of cystyl aminopeptidase were demonstrated by *Page* et al. (157) in pregnancy sera, which appear to have similar substrate specificities (156, 158). *Sjöholm & Yman* (159) found that the two forms were of the same size. The two serum cystyl aminopeptidases elute together on gel filtration (39). These findings are in agreement with those of *Sanderink* et al. (40) who showed that the two forms have an identical molecular mass on 2-dimensional electrophoresis gels, but differ in isoelectric point.

## Clinical Significance of Aminopeptidases in Serum

### Alanine aminopeptidase

Evaluations of the clinical value of the analysis of alanine aminopeptidase in serum were mainly carried out in the early sixties, after initial reports of *Goldbarg & Rutenburg* (91, 160) that (what now may be identified as) alanine aminopeptidase was a specific marker for carcinoma of the head of the pancreas. Subsequently they also found elevated alanine aminopeptidase activities in cases of biliary and hepatic tumours, intra- and extrahepatic obstruction and drug-induced hepatitis (161). Others found that serum alanine aminopeptidase was elevated in cases of pancreas carcinoma only when accompanied by biliary obstruction (162, 163). It appears that increased alanine aminopeptidase concentrations are generally found in cases of intra- and extrahepatic cholestasis, malignant hepatobiliary disease and alcoholic liver disease (23, 164–166). γ-Glutamyltransferase, which is more sensitive, and also alkaline phosphatase are now often preferred for purposes of diagnostic screening for hepatobiliary disease.

Although alanine aminopeptidase elevations are rather specific for cases in which obstruction occurs – possibly because it is specifically localized in canalicular membranes (27, 129) – the value of a single alanine aminopeptidase measurement in the differential diagnosis of liver disease is poor. Yet, both *Neef* et al. (167) and *Crofton* et al. (168) found by the use of multivariant analysis that alanine aminopeptidase was a useful discriminator when associated with other parameters. In this matter alanine aminopeptidase is more efficient than γ-glutamyltransferase, the elevations of which in serum are rather unspecific.

There are few recent data on the biological variations of alanine aminopeptidase in serum. Alanine aminopeptidase activities increase in childhood and decrease after puberty (101). Alanine aminopeptidase activities are higher in men than in women (100, 101, 105). Activity in serum may be increased by smoking, alcohol consumption or drug intake. Hence, it appears possible that serum alanine aminopeptidase is influenced by microsomal enzyme induction in the liver, similarly to γ-glutamyltransferase (101, 169).

*Alanine aminopeptidase isoforms*

Interest in the determination of alanine aminopeptidase isoforms in serum arose partly because of a report by *Beier* et al. (170). They separated two alanine aminopeptidase bands from serum by agar gel electrophoresis and stated that the intensity of one band correlated with cholestasis, while the other one was a sensitive and specific marker for pancreatic disease. However, *Schlaeger & Kattermann* (171) found 40% of false negative results in cases of pancreatic disease. The appearance of a second alanine aminopeptidase band was related to the presence of cholestasis. Several other groups using agar gels or polyacrylamide gels as support media obtained similar results; additional slower moving alanine aminopeptidase bands are generally found when cholestasis is present or may be suspected (23, 106, 124, 130).

Early studies also showed slower moving alanine aminopeptidase bands in sera of subjects taking oral contraceptive drugs or anticonvulsant drugs (172, 173). No indication was found whether this was due to liver damage or enzyme induction. *Phillips & Manildi* (174) found additional alanine aminopeptidase bands on cellulose-acetate membranes in cases of metastatic disease, sometimes without signs of liver involvement.

Most authors have concluded that the fact that additional bands appear is of no value for a differential diagnosis of hepatobiliary diseases. The large number of additional alanine aminopeptidase bands in hepatobiliary disease, the lack of their characterization in early studies and the range in analytical methods has rendered interpretations and comparisons of alanine aminopeptidase patterns impossible. But now that the multiple forms have become better characterized — and thus easier to identify — it is possible that specific subfractions of alanine aminopeptidase and other liver enzymes may become of diagnostic interest. For example, *Wenham* et al. (132) reported recently that some HDL-γ-glutamyltransferase complexes are predominant in patients with extrahepatic jaundice. *Sanderink* et al. (41) found that the F (fast) form of hydrophilic alanine aminopeptidase separated on polyacrylamide electrophoresis gels may be a sensitive marker of alcohol abuse.

However, the development of more quantitative methods of analysis of these isoforms appears necessary in order to perform a definitive evaluation of their clinical interest and to initiate a still hypothetical large scale application in routine laboratory medicine.

## Leucine aminopeptidase

Leucine aminopeptidase is a liver cytosolic enzyme, and therefore a marker for hepatic cell lysis (8). Some authors have even stated that leucine aminopeptidase is a more sensitive marker for acute hepatitis than the aminotransferases (81, 175). *Kanno* et al. (84) compared leucine aminopeptidase values for different groups of patients with hepatic diseases. The highest activities of leucine aminopeptidase in serum were found for patients with acute hepatitis with different causes or with embolized hepatoma. Relatively high increases were found in sera from patients with prolonged or chronic hepatitis, cirrhosis, or non-embolized hepatoma. Only slight elevations were noticed in cases of obstructive disease or microsomal enzyme induction. *Pancheva-Haschen & Haschen* (175) observed relatively elevated leucine aminopeptidase values as compared to transaminase levels in patients with active cytomegalovirus infections. They subsequently found that leucine aminopeptidase, alanine aminopeptidase and γ-glutamyltransferase activities were higher in lymphoid cells than in normal lymphocytes. It was concluded that serum leucine aminopeptidase may be a marker for plasmacytoid lymphocyte proliferation in acute viral infections.

## Cystyl aminopeptidase

Serum cystyl aminopeptidase levels increase during normal pregnancy until shortly before the onset of labour (119, 176, 177). Cystyl aminopeptidase activity in amniotic fluid shows a decrease during pregnancy (53). Whether a shift in the oxytocin/cystyl aminopeptidase ratio induces the onset of labour remains hypothetical (57, 119).

The fact that cystyl aminopeptidase is produced by the placenta has led to studies on its utility as a placental function test. Effectively, serum cystyl aminopeptidase levels are statistically significantly lower in chronic placental insufficiency or before a premature birth (166, 178, 179). However, other placental function tests, i. e. human placental lactogen and oestriol are preferred for this purpose (180). Moreover,

the interest of placental function tests as a whole has been questioned recently, because of too high a level of false positive and false negative results (180).

Cystyl aminopeptidase also has angiotensinase activity and relatively high levels of the enzyme have been found in pregnancy sera in cases of pre-eclampsia and low levels in the late stage of severe pre-eclampsia (181). Serum activities of aspartyl aminopeptidase (aminopeptidase A), which also exhibits angiotensinase activity, show a significant decrease in cases of pre-eclampsia (182). Cystyl aminopeptidase levels have also been reported to be increased in sera of women with ovarian adenocarcinomas, but this has not been confirmed (183, 184).

### Tripeptide aminopeptidase

The clinical usefulness of tripeptide aminopeptidase has been investigated only very recently by *Kanda* et al. (66, 185). Because tripeptide aminopeptidase is mainly localized in the cytosolic subfraction of the liver, the activity in sera of patients with liver disease correlates well with those of alanine aminotransferase. An exception to this is found in hepatoma, in which tripeptide aminopeptidase shows a relatively greater increase. This was explained by a changed tripeptide aminopeptidase/alanine aminotransferase ratio in cancerous tissues (85). Further, preliminary results showed an increase of tripeptide aminopeptidase in some patients with leukaemias or autoimmune diseases, possibly related to the high tripeptide aminopeptidase activities that are present in leukocytes.

## Conclusion

Studies on the biological functions of aminopeptidases and on the value of their determination in clinical chemistry have been complicated by the heterogeneity of these enzymes, with overlapping specificities for both biological and synthetic substrates, and by the lack of characterization of the individual enzymes. Further data are also required on their multiple forms, especially for alanine aminopeptidase.

Aminopeptidases have now been purified from various tissues and characterized, and this work is still in progress. Circulating multiple forms have also been better characterized, but their origin is still unclear.

These developments should lead to more specific, quantitative assays, which may increase our understanding of the biological roles of aminopeptidases, provide an aid to the study of pathological processes and give a better insight in their significance in clinical chemistry.

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