Purification and Partial Characterization of Barley Leucine Aminopeptidase

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ABSTRACT

A peptidase acting on Leu-Gly-Gly and Leu-Tyr at pH 8 to 10 was purified about 670-fold from germinated grains of barley (Hordeum vulgare L.). Gel electrophoretic analyses indicated a purity of about 90%. The purified enzyme is remarkably similar to mammalian leucine aminopeptidases (EC 3.4.1.1) both in chemical and in enzymatic properties. It has a sedimentation constant of 12.7S and a molecular weight of about 260,000. The enzyme has a high activity on leucine amide and di- and tripeptides with N-terminal leucine or methionine; leucyl-β-naphthylamide, in contrast, is hydrolyzed very slowly. The enzyme also liberates N-terminal amino acids from the insulin B chain. The pH optima for the hydrolysis of different substrates depend on the buffers used; highest reaction rates are generally obtained at pH 8.5 to 10.5. Mg²⁺ and Mn²⁺ ions stabilize (and probably activate) the enzyme. In contrast to mammalian leucine aminopeptidases, the barley enzyme is inactivated in the absence of reducing sulfydryl compounds.

This paper gives an account of the isolation and properties of the Leu-Gly-Gly hydrolyzing enzyme of germinating barley grains. The results show that the enzyme is different from the various other known barley peptidases (21) and that it is remarkably similar to the mammalian (and bacterial) leucine aminopeptidases.

MATERIALS AND METHODS

Plant Material. The barley (Hordeum vulgare L.) samples used were Pirkka (a Finnish six-row cultivar) “high enzyme” malt and Ingrid (a Swedish two-row cultivar) barley, obtained from Oy Lahden Polttimo, Lahti, Finland.

Reagents. The following enzyme substrates were obtained from Mann Research Laboratories: L-Leu-L-Tyr, L-Leu-Gly, L-Leu-Gly-Gly, L-Met-L-Leu-Gly, L-leucine amide hydrochloride, L-Leu-β-naphthylamide, benzoyl-β-arginine-p-nitroanilide, and porcine insulin carboxymethylated B chain. L-Ala-Gly was purchased from the Sigma Chemical Co. Gentamicin fast garnet GBC salt was obtained from I.C.I. Organics Inc., 2, 4, 6-trinitrobenzene sulfonic acid from BDH Chemical Ltd., di-thiothreitol (A grade) from Calbiochem Inc., and disopropylfluorophosphate from the Sigma Chemical Co. DEAE-cellulose was supplied by the Carl Schleicher & Schuell Co. and Sephadex G-200 by Pharmacia. All the common reagents were of analytical grade unless otherwise stated.

Enzyme Assay. The assay was a modification of earlier methods in which the amino acids liberated by a peptidase are estimated with TNBS reagent in the presence of cupric ions to block the reaction of the peptide amino group with TNBS (3, 21). The substrate solution contained 6.25 mM Leu-Tyr in sodium carbonate buffer of pH 9.2, ionic strength 0.1, with 0.1 mM DTT. The enzyme solutions were diluted with 10 mM MgSO₄, containing 0.1 mM DTT. In a typical assay, 100 μl of substrate was mixed with 25 μl of diluted enzyme (final concentrations 5 mM Leu-Tyr, 2 mM Mg²⁺), the mixture was incubated at 30 C for 30 min, and the reaction was terminated by addition of 3 ml of fresh TNBS reagent, prepared by mixing 5% (w/v) sodium tetraborate-2.5 mM cupric sulfate-0.2% (w/v) TNBS (3:2:1). After 60 min at 30 C, 1 ml of 1 N acetic acid was added and the absorbances were read at 340 nm. The activities are expressed as enzyme units, 1 unit (U) corresponding to the hydrolysis of 1 μmole of substrate/min at 30 C.

Hydrolysis of Other Substrates. The rates of enzymatic hydrolyses of other di- and tripeptides were determined with the method used for Leu-Tyr.

In 1929 Linderström-Lang and Sato (16) reported the presence of two different peptidases both in swine intestinal mucosa and in germinating barley grains; one of the enzymes liberated N-terminal leucine from Leu-Gly-Gly optimally at pH 8.6, the other hydrolyzed Ala-Gly with a pH optimum at 7.8. The first enzyme has been purified from several mammalian tissues (7), the most thoroughly characterized preparations being those from swine kidney (27, 28) and bovine ocular lens (10, 11). All of these purified enzymes resemble each other closely; the mol wt reported are in the range 250,000 to 330,000, and the enzymatic properties are almost identical. They are generally called leucine aminopeptidases, EC 3.4.1.1. A peptidase almost identical with the mammalian leucine aminopeptidases has been isolated from Escherichia coli (31).

Similar enzyme activities (hydrolysis of Leu-Gly-Gly or Leu-NH₂ at pH 8–10) have also been detected in other higher plants besides barley: the leaves of spinach and cabbage (2), and the germinating seeds of Pinus pinea L. (9) and Cucurbita maxima L. (1). However, none of these enzymes has yet been extensively purified and characterized.

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3 Abbreviations: TNBS: 2, 4, 6-trinitrobenzene sulfonic acid, BAPA: benzoyl-β-arginine-p-nitroanilide; p-CMB: p-chloromercuribenzoic acid; DTT: dithiothreitol; 2-ME: 2-mercaptoethanol; -NA: naphthylamide.
The hydrolyses of Leu-NH₂, Leu-β-NA, and insulin B chain were also carried out in sodium carbonate buffer, pH 9.2, containing 2 mM Mg²⁺ and 0.1 mM DTT. For Leu-β-NA the substrate concentration was 0.6 mM and the liberated β-naphthylamine was determined by a standard method (14). Leu-NH₂ was used at 10 mM concentration, the reaction was terminated by addition of an equal volume of 0.2 M sodium citrate buffer, pH 2.2, and the liberated leucine was determined with an amino acid analyzer (Hitachi-Perkin-Elmer liquid chromatograph 034, Ligand method for protein hydrolysates). In the hydrolysis of the insulin B chain, the substrate concentration was 2 mM, and the reaction products were identified by TLC (14).

Universal buffer (17) was used in some experiments; however, to prevent chelation of Mg²⁺ the citric acid was replaced by equimolar acetic acid.

Estimation of Protein. The absorbance at 280 nm was generally used as a measure of protein concentration; in the calculations it has been assumed that an absorbance of 1 corresponds to a protein concentration of 1 mg/ml. The protein contents of crude extracts were assayed with the biuret reaction (13) with BSA as a standard.

Electrophoreses. Anodic disc electrophoreses in 7.5% polyacrylamide gels at pH 9.5 were made with the buffer system of Ornstein and Davis (6). Electrophoresis in a gel concentration gradient (gradipore electrophoresis) was performed at pH 8.4 (18, 26); the equipment and gels were obtained from Universal Scientific Ltd., London. The swine kidney leucine aminopeptidase and human serum albumin used as reference proteins were supplied by Worthington Biochemical Corp. and Ab Kabi, Stockholm, respectively.

Ultracentrifugation. Ultracentrifugations were made with a Spinco Model E analytical ultracentrifuge using UV-visible scanning optics with the monochromator set at 280 nm.

RESULTS

PRELIMINARY EXPERIMENTS ON STABILITY OF ENZYME

Compared with various other barley peptidases, the enzyme (Leu-Tyr hydrolyzing activity at pH 9.2) was remarkably stable at high pH values (Fig. 1). The activity of crude extract decreased only about 10% during 1 hr at pH 10, 30°C, whereas the two "naphthylamidases" acting on Leu-β-NA (14) were completely inactive, and the activities on Ala-Gly and BAPA decreased by about 40%. The extract incubated at pH 10 was also completely inactive in the hydrolysis of Arg-β-NA. This result indicates that the third barley "naphthylamidase" (14) was also inactivated at this pH. On the acid side all the enzymes were simultaneously inactivated below pH 5.

The effects of temperature on the crude enzyme were studied at pH 9. The 55 to 65% ammonium sulfate fraction of malt extract was extensively dialyzed against 20 mM tris-HCl buffer, pH 9, containing 5 mM 2-ME, 10 mM MgSO₄, and 5% (v/v) glycerol; small samples of the dialyzed solution were incubated at different temperatures for 15 min. No inactivation occurred at temperatures below 75°C; at 80°C the activity decreased about 20% and at 85°C about 60%.

In spite of its remarkable stability at high pH or temperature, the enzyme was slowly inactivated at room temperature at pH 7 to 9, especially in dilute solutions. The inactivation was significantly retarded by addition of 10 mM MgSO₄, 5 mM 2-ME, and 5% (v/v) glycerol to the solutions (data not shown).

PURIFICATION

Pirkka "high enzyme" malt was selected as the starting material because both total and specific activities were severalfold higher in the malt than in samples of ungerminated barley. In most of the purification steps, tris-HCl buffers of pH 7.5 were used; the buffers contained 10 mM MgSO₄, 5 mM 2-ME, and 5% (v/v) glycerol. Unless otherwise stated, the operations were carried out in a cold room with a temperature of about 7°C.

Extraction. The malt was ground in a Wiley mill (1-mm sieve), and 7 kg of ground malt were suspended in 14 liters of H₂O containing 25 mM 2-ME. The extraction was continued for 2 hr at room temperature with occasional stirring. The extract was separated by centrifugation at 5°C, its pH was adjusted to 6.3 with 1 N NaOH, and it was left standing overnight at 7°C.

Treatment at pH 10. The pH of the cold extract was adjusted to 10 with 1 N NaOH. The solution was then warmed slowly (15 min) to 30°C and incubated for 1 hr at this temperature. The pH was again adjusted to pH 6.3 with 2 N acetic acid. The heavy precipitate that formed was not removed before the next step.

Fractional Precipitation with Ammonium Sulfate. Solid ammonium sulfate (practical grade) was added to the suspension to give 55% saturation; when the salt had dissolved, the pH was adjusted to 6 with 1 N NaOH, and the suspension was incubated at room temperature for 1 hr. The inactive precipitate was removed by centrifugation at 5°C, and solid ammonium sulfate was added to the supernatant to give 35% saturation. After 1 hr at room temperature the active precipitate was collected by centrifugation at 5°C. The precipitate was suspended in the 20 mM pH 7.5 buffer to give a volume of 180 ml. The solution was dialyzed extensively against the same buffer, and an inactive dark brown precipitate was removed by centrifugation.

Adsorption on DEAE-cellulose. The enzyme solution (about 250 ml) was passed through a 4 × 12.5 cm column of DEAE-cellulose equilibrated with the 20 mM pH 7.5 buffer, and the column was washed with 700 ml of the 20 mM buffer and 300 ml of 50 mM pH 7.5 buffer. The enzyme, which was adsorbed while the bulk of the inactive proteins passed through the column, was eluted as a sharp peak with 110 mM pH 7.5 buffer. The crude enzyme was concentrated by precipitation.
with ammonium sulfate (80% saturation, 4 hr). The precipitate was dissolved in 7.5 ml of 50 mM buffer.

**Chromatography on Sephadex G-200.** The active solution from the preceding step was fractionated in a column of Sephadex G-200 in the 50 mM pH 7.5 buffer (Fig. 2). The enzyme was eluted close to the void volume of the column before the major protein peak. The active fractions were combined, the enzyme was concentrated by ammonium sulfate precipitation as before, and the precipitate was dissolved in 4 ml of the 50 mM buffer.

**Acetone Precipitation.** The active solution was cooled to 0 C, and an equal volume of cold (−20 C) acetone was slowly added while the suspension was gradually cooled to −20 C. After 30 min, the small active precipitate was collected by centrifugation at −20 C and dissolved in 4 ml of cold 50 mM buffer.

**Storage of the Enzyme Preparation.** The enzyme was found to be relatively stable in 50% glycerol. Therefore, an equal volume of glycerol was added to the solution (4 ml), and this solution was stored at −18 C. Slow inactivation occurred even under these conditions, and only about 20% of the activity was left after storage for 1 year.

The results of the purification are summarized in Table I. The final preparation contained 21.4 mg of protein with a specific activity of 564 U/mg. The next section shows that the purity of the preparation was about 90%. From the activity of the extract and the specific activity of the purified enzyme, the amount of the enzyme in the malt sample was calculated to be about 16 mg/kg.

**PURITY OF PREPARATION**

The purified enzyme was subjected to anodic disc electrophoresis at pH 9.5 and to gradipore gel electrophoresis at pH 8.4. In both runs (Fig. 3 and 4) a single major protein band was

**Fig. 2.** Gel chromatography of the partly purified enzyme on Sephadex G-200. The concentrated active fraction from DEAE-cellulose adsorption (7.5 ml) was chromatographed in a 2.5 × 83 cm column of Sephadex G-200 in 50 mM tris-HCl buffer, pH 7.5, containing 5 mM 2-ME, 10 mM MgSO4, and 5% glycerol; the run was made at +7 C with a flow rate of 20 ml/hr; A280 (●) and Leu-Tyr hydrolyzing activity (○) were determined from 5-ml fractions of the eluate.

**Table 1. Purification of Enzyme**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>8570</td>
<td>8570a</td>
<td>12000</td>
<td>0.84a</td>
<td>100</td>
</tr>
<tr>
<td>Extract after treatment at pH 10</td>
<td>9950</td>
<td></td>
<td>72400</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>55-65% ammonium sulfate precipitate</td>
<td>180</td>
<td>9750</td>
<td>64500</td>
<td>6.61</td>
<td>89</td>
</tr>
<tr>
<td>Same after dialysis</td>
<td>250</td>
<td>5410</td>
<td>45600</td>
<td>8.4</td>
<td>63</td>
</tr>
<tr>
<td>Pooled DEAE-cellulose fractions</td>
<td>62</td>
<td>238</td>
<td>23800</td>
<td>100</td>
<td>33</td>
</tr>
<tr>
<td>Pooled Sephadex G-200 fractions</td>
<td>37.5</td>
<td>31.5</td>
<td>18100</td>
<td>600</td>
<td>25</td>
</tr>
<tr>
<td>50% acetone precipitate</td>
<td>4</td>
<td>16.4 (21.4a)</td>
<td>12040</td>
<td>745 (564a)</td>
<td>17</td>
</tr>
</tbody>
</table>

1 Estimated from A280.
2 Biuret reaction.

**Fig. 3.** Anodic disc electrophoresis of the enzyme in 7.5% polyacrylamide gel at pH 9.5. A: Partially purified enzyme before chromatography on Sephadex G-200, 200 μg; B: final enzyme preparation, 40 μg.

**Fig. 4.** Gradipore electrophoresis of the purified enzyme (A) and a sample of human serum albumin containing a series of oligomers (B, C). A concave gel gradient from 4 to 27% acrylamide, pH 8.4, running time 64 hr at 110 V, +7 C; 62 μg of purified enzyme (A) and 150 and 75 μg serum albumin (B, C) were applied to the sample slots. The monomer of serum albumin migrates out of the gel in the conditions used; the positions of the dimer, trimer, etc. have been marked 2X, 3X, etc.
visible. In disc electrophoresis there were two weak bands behind the main fraction; in the gradipore gel three minor bands were visible.

The gradipore technique separates proteins according to molecular size, whereas in disc electrophoresis both electric charge and molecular size affect the mobility. Because a single major band was obtained in both runs, it is most likely that the preparation contained a single major component. In addition, there were three minor impurities which according to visual estimation, accounted for less than 10% of the total protein.

**CHEMICAL PROPERTIES**

In the gel filtration on Sephadex G-200 (Fig. 2), the enzyme was eluted near the void volume of the column. This fact indicates a high mol wt in the range 200,000 to 300,000. A more accurate estimate was obtained by comparing the migration distance of the enzyme in gradipore electrophoresis with those of human serum albumin oligomers (Fig. 4). A linear plot of migration distance versus the logarithm of mol wt was obtained for the oligomers, and the position of the enzyme corresponded to a mol wt of 260,000. The migration of the barley enzyme was also compared with that of commercial swine kidney leucine aminopeptidase. The swine enzyme migrated about 2 mm further (44 versus 42 mm); this corresponded to a mol wt of 240,000.

The enzyme preparation was subjected to analytical ultracentrifugation in 50 mM tris-HCl buffer, pH 7.5, containing 10 mM MgSO₄ and 0.1 mM DTT at three different concentrations; the rotor speed was 42,040 rpm and the temperature 4°C. A single sedimenting boundary was observed in each case, the s₂₀,₅₀ values obtained being 12.76S (A₂₉₀ = 0.8), 12.61S (A₂₉₀ = 0.4), and 12.66S (A₂₉₀ = 0.2). The mean value is 12.7S.

**ENZYMATIC PROPERTIES**

The experimental on the catalytic properties of the enzyme were performed with a preparation which had been stored in 50% glycerol at -18°C for about 1 year. At that time the specific activity was 104 U/mg, corresponding to about 20% of the original activity.

**Substrate Specificity.** The rates of hydrolysis of some peptidase substrates by the enzyme preparation at pH 9.2 are listed in Table II. Di- and tripeptides with N-terminal leucine or methionine are all rapidly hydrolyzed; Leu-NH₂ is a good substrate as well; very slow reaction rates are observed for Leu-β-NA and Ala-Gly, and BAPA is not hydrolyzed at all. The effects of substrate concentration on reaction rate were studied with four of the substrates. The plots of [S]/v versus [S] were linear and gave the following Km values: Leu-Tyr 0.16 mM, Leu-Gly 9.6 mM, Met-Leu-Gly 0.16 mM, and Leu-β-NA 0.16 mM.

The enzyme preparation also liberated N-terminal amino acids from the insulin B chain. After incubation for 8 hr (2 mM substrate, pH 9.2, 27 U of enzyme in a volume of 0.5 ml), the reaction mixture gave five amino acid spots in TLC; the two most intense spots had R₅ values identical with those of phenylalanine and valine, the two N-terminal amino acid residues of the B chain.

**Effects of pH on Reaction Rate.** The pH versus activity curves for the hydrolysis of Leu-Tyr in five different buffers are shown in Figure 5. The buffer ions had pronounced effects both on the pH optimum and on the maximal reaction rate. The highest rate was obtained in unbuffered reaction mixture at pH 9.5; in the universal buffer the optimum pH was above pH 11. The cation in all the buffers was Na⁺ (20–100 mM in most cases). Tests with NaCl showed that Na⁺ (and Cl⁻) had only minimal effects on the activity over the concentration range 20 to 200 mM (pH 9); this suggests that the buffer effects are due mainly to the respective anions.

Figure 6 shows corresponding data for two other substrates, Leu-Gly-Gly and Leu-β-NA (modified universal buffer). The curves are very similar to the corresponding curve for Leu-Tyr.

**Effects of Some Activators and Inhibitors.** During the development of the purification method, it was noted that the enzyme is stable in the absence of a sulfhydryl compound (DTT or 2-ME) and Mg⁺⁺ or Mn⁺⁺. A quantitative experiment with a dilute solution of the purified enzyme is shown in Figure 7. Rapid inactivation occurs in the absence of DTT and MgSO₄; either compound alone gives partial protection, and in the presence of both DTT and Mg⁺⁺ the inactivation is further slowed down.

At higher concentrations dithiothreitol also caused partial inactivation of the enzyme, which had lost about 80% of its

![Figure 5. Effects of the pH on the hydrolysis of Leu-Tyr by the purified enzyme in different buffers.](https://www.plantphysiol.org/doi/fig/10.1104/pp.55.1.812)

![Figure 6. Table II. Hydrolysis of Different Substrates by Purified Enzyme at pH 9.2.](https://www.plantphysiol.org/doi/fig/10.1104/pp.55.1.812)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction Rate (μmoles/min·mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-Tyr</td>
<td>104</td>
</tr>
<tr>
<td>Leu-Gly</td>
<td>87</td>
</tr>
<tr>
<td>Ala-Gly</td>
<td>1.5</td>
</tr>
<tr>
<td>Leu-Gly-Gly</td>
<td>168</td>
</tr>
<tr>
<td>Met-Leu-Gly</td>
<td>146</td>
</tr>
<tr>
<td>Leu-NH₂</td>
<td>27</td>
</tr>
<tr>
<td>Leu-β-NA</td>
<td>0.10</td>
</tr>
<tr>
<td>BAPA¹</td>
<td>0.0</td>
</tr>
</tbody>
</table>

¹ Standard assay for BAPA hydrolysis at pH 10.4.
original activity during the storage. The rate and extent of the activation were dependent on both pH and buffer composition; generally, activations of about 2-fold were obtained with 0.5 to 5 mM DTT. After the activation, however, the enzyme was markedly more labile than the unactivated preparation. Therefore, all the experiments concerned with the other catalytic properties of the enzyme were made without pre-activation with DTT.

p-CMB, a potent inhibitor of enzymes with —SH groups at their active sites, did not markedly inhibit the enzyme. On the contrary, it had definite stabilizing effects on the enzyme pre-activated with DTT. The results of a typical experiment are shown in Table III. In the tris-HCl buffer at pH 7, 5 mM p-CMB protected the enzyme even better than 0.5 mM DTT.

Disisopropylfluorophosphate did not have any effect on the activity even at 1 mM concentration (pH 7, 5 mM 2-ME, 10 mM MgSO₄, + 7 C, 1 hr).

![Figure 6](image)

**Figure 6.** Effects of pH on the hydrolysis of Leu-Gly-Gly and Leu-β-NA by the purified enzyme in modified universal buffer. Substrate concentrations 5 mM Leu-Gly-Gly and 0.6 mM Leu-β-NA. Note the difference between the two activity scales.

![Figure 7](image)

**Figure 7.** Effects of DTT and Mg²⁺ on the stability of the enzyme. The purified enzyme was preactivated with 1 mM DTT (sodium carbonate buffer pH 9.2, 30 min at 30 C) and diluted 200-fold to 20 mM tris-HCl buffers, pH 7, containing the compounds shown on the figure at the following concentrations: DTT, 0.5 mM; MgSO₄, 10 mM; EDTA, 5 mM. The solutions were incubated at 23 C or 7 C and assayed for Leu-Tyr hydrolyzing activity in the absence of added DTT or Mg²⁺.

### Table III. Effects of p-CMB and DTT on Stability of Enzyme Preactivated with DTT

<table>
<thead>
<tr>
<th>Activity</th>
<th>Treatment</th>
<th>Sodium carbonate buffer, pH 9.2, 30 min at 30 C</th>
<th>Tris-HCl buffer, pH 7, 20 min</th>
<th>5 min</th>
<th>30 min</th>
<th>135 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 min</td>
<td>30 min</td>
<td>135 min</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>38</td>
<td>6</td>
<td>39</td>
</tr>
<tr>
<td>5 mM p-CMB</td>
<td></td>
<td></td>
<td></td>
<td>91</td>
<td>14</td>
<td>150</td>
</tr>
<tr>
<td>0.5 mM DTT</td>
<td></td>
<td></td>
<td></td>
<td>161</td>
<td>89</td>
<td>138</td>
</tr>
</tbody>
</table>

### DISCUSSION

Barley grain extracts catalyze the hydrolysis of Leu-Gly-Gly (and Leu-Tyr) at pH 8 to 10. We have purified this activity about 670-fold (on protein basis). Gel electrophoretic separations indicate that about 90% of the preparation consists of a single protein, which we believe is the enzyme responsible for the activity. The enzymatic and some of the chemical properties of the enzyme were determined using this “90% pure” preparation.

There are three main questions concerning the data presented. Does the enzyme differ from the other known barley peptidases? Is the preparation sufficiently pure for studies on the catalytic properties of the enzyme? To what extent does the barley enzyme resemble mammalian leucine aminopeptidases?

The enzyme differs from all the other peptidases of barley (21, 25) in its large molecular size (mol wt about 260,000); the other known peptidases have mol wt in the range 50,000 to 100,000. In addition, it differs from most of the other enzymes in several properties; only a few of the most marked differences are listed below:

The three carboxypeptidases of barley (23, 24, 30) are all inactivated by disisopropylfluorophosphate), which did not have any effect on the present enzyme. The three “naphthylamidases” of barley (14) are highly active on aminoacyl-β-naphthylamides at pH 7.2. The present enzyme showed only minimal activity on Leu-β-NA with a pH optimum near pH 11. Finally, the very low activity of the enzyme on Ala-Gly differentiates it from the peptidase acting on this substrate.

Burger et al. have purified three peptidases from germinated barley and called them barley peptidase hydrolyse A or BAPase, peptide hydrolyse B or BAAEase (4), and peptide hydrolyse C or ANase IV (5). Our enzyme does not hydrolyze BAPA at all. Furthermore, we have shown that peptide hydrolyse B is actually identical with the carboxypeptidase purified by Mikola and Pietilä (22). Finally, the elution volume on Sephadex G-100 (5) indicates that hydrolyse C has a relatively low mol wt, certainly below 100,000. Accordingly, we conclude that the purified enzyme is different from all the other nine barley peptidases, which have been characterized to some extent.

The second question is more difficult. The purification procedure includes two steps in which most of the other peptidases are removed. The “naphthylamidases” and the carboxypeptidase purified by Visuri et al. (30) are inactivated at pH 10 (Fig. 1 and ref. 30). Secondly, all enzymes with mol wt below 100,000 are in principle eluted definitely later from columns of Sephadex G-200. Therefore, it seemed most unlikely that...
the preparation could contain more than traces of the other peptidases, and direct activity determinations were made with only two substrates; BAPA was not hydrolyzed at all, and the pH versus activity curve for the hydrolysis of Leu-β-NA did not have even a shoulder at pH 7.2. In conclusion, we think that the enzyme preparation did not contain significant quantities of the other peptidases, and that the data on the catalytic properties of the preparation reflect the properties of a single enzyme.

The three most thoroughly studied leucine aminopeptidases are the enzymes from swine kidney (27, 28), bovine ocular lens (10, 11), and Escherichia coli (31). The characteristic properties of these enzymes include high mol wt (250,000–330,000), stability at high temperatures (up to 70°C) and at high pH values, stabilization or activation by Mg²⁺ and Mn²⁺, and pH optima at pH 8 to 10. The substrate specificities are quite similar as well: di- and tripeptides with N-terminal leucine and leucine amide are all rapidly hydrolyzed, but Leu-β-NA is a poor substrate. The enzyme from swine kidney also liberates N-terminal amino acids from proteins (12) (data are not available for the other enzymes). The barley peptidase has all the properties listed above. A particularly striking similarity concerns the sedimentation constant, which can generally be determined more accurately than mol wt. The following S₂₀,w values have been reported for the enzymes from the three respective sources: swine kidney 12.6 S (28) and 12.3 to 13.4 S, depending on buffer (20); bovine ocular lens 12.6 S (15) and 12.9 S (19); and E. coli 12.0 S (31). The value obtained for the barley enzyme was 12.7 S.

The only difference observed between the barley enzyme and the well known leucine aminopeptidases concerns the stabilizing effects of sulfydryl compounds. The barley peptidase is inactivated in the absence of DTT or 2-ME; no corresponding requirement has been observed for the other enzymes. However, the barley enzyme cannot be a "true" sulfydryl enzyme with a reacting —SH group at the active center, as it is not inactivated by p-CMB. In contrast, the unusual responses to p-CMB (stabilization in certain conditions) seem to be a feature shared at least by the enzyme from the ocular lens. This enzyme contains 9 to 10 —SH groups, and when they are titrated with p-CMB, an initial decrease in activity is followed by activation; complete titration, finally, causes an inhibition of about 50% (8).

In conclusion, the barley enzyme is very similar to the mammalian (and bacterial) leucine aminopeptidases in both chemical and catalytic properties. Therefore, we suggest that it should be called barley leucine aminopeptidase. We would like to stress that the suggested name does not imply specificity for N-terminal leucyl residues but resemblance to a group of enzymes traditionally called leucine aminopeptidases.

In a previous paper (21) we reported that in germinating barley seeds Leu-Tyr hydrolyzing activity is present in all living tissues: the aleurone layer of the endosperm, the scutellum, and the growing tissues of the seedling. As mentioned in the introduction, corresponding activity has been detected in several other plant tissues, including both dicots and a gymnosperm (Pinus pinea L.). However, none of these enzymes has been extensively characterized, and it is not definitely known whether they are "true" leucine aminopeptidases. The data on the barley enzyme are quite suggestive, however, indicating that leucine aminopeptidases possibly are universal enzymes, as ubiquitous in plant cells as in mammalian tissues.

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