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Optimization of the Production of a Honeybee Odorant-Binding Protein by *Pichia pastoris*

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A honeybee putative general odorant-binding protein ASP2 has been expressed in the methylotrophic yeast *Pichia pastoris.* It was secreted into the buffered minimal medium using either the α-factor preprosequence with and without the Glu-Ala-Glu-Ala spacer peptide of *Saccharomyces cerevisiae* or its native signal peptide. Whereas ASP2 secreted using the α-factor preprosequence with the spacer peptide showed N-terminal heterogeneity, the recombinant protein using the two other secretion peptides was correctly processed. Mass spectrometry showed that the protein secreted using the natural peptide sequence had a mass of 13,695.1 Da, in perfect agreement with the measured molecular mass of the native protein. These data showed a native-like processing and the three disulfide bridges formation confirmed by sulfhydryl titration analysis. After dialysis, the recombinant protein was purified by one-step anion-exchange chromatography in a highly pure form. The final expression yield after 7-day fermentation was approximately 150 mg/liter. To our knowledge, this is the first report of the use of a natural insect leader sequence for secretion with correct processing in *P. pastoris.* The overproduction of recombinant ASP2 should allow ligand binding and mutational analysis to understand the relationships between structure and biological function of the protein.

In vertebrates, odorant-binding proteins (OBPs)[2] are soluble, low-molecular-weight proteins secreted by different nasal glands into the olfactory mucus. These very abundant proteins are involved in perception of odorants (1–3). Although their functions are still unclear, OBPs are thought to be involved in the solubilization, transport, and/or deactivation of odorants (4). In insect antennae, soluble proteins, distributed in the sensillum lymph surrounding the dendrites of olfactory sensilla, showed similar biochemical properties (5). Insect OBPs are classified as pheromone-binding proteins (PBPs) and general odorant-binding proteins (GOBPs). While PBPs are involved in sex pheromone detection, GOBPs seem to play a more general role in olfaction (6). However, to date, only one study has described the binding properties of insect GOBPs (7). Recently, a putative GOBP, called ASP2 for antennal-specific protein, specifically expressed in olfactory antennal tissues of the honeybee, *Apis mellifera* L., has been purified, characterized and cloned (8). ASP2 is a nonglycosylated protein of 13,695.2 ± 1.6 Da (calculated 13,694.8) with three disulfide bridges and a calculated isoelectric point of 4.8. Its higher expression level in the antennae of workers than in drones suggests some involvement in general odorant detection (9). To study the biological properties and functions of ASP2 in olfaction, a ready supply of protein is required and therefore attempts to develop an expression sys-

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[2] Abbreviations used: *AOX1,* gene encoding AOXI alcohol oxidase; ASP2, honeybee antennal-specific protein; BMGY, buffered minimal glycerol medium; BMM, buffered minimal methanol; GOBP, general odorant-binding protein; *HIS4,* gene encoding HIS4 histidinol dehydrogenase; HPLC, high-performance liquid chromatography; IS-MS, ion spray-mass spectrometry; *KEX2,* gene encoding Kex2 protease; MD, minimal dextrose medium; MM, minimal methanol medium; Mut^s, methanol utilization slow; OBP, odorant-binding protein; PBP, pheromone-binding protein; PCR, polymerase chain reaction; *PHOI,* gene encoding PHOI acid phosphatase; QAE, quaternary amino ethyl; RDB, regeneration dextrose medium; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; *STE13,* gene encoding Ste13 protease; YNB, yeast nitrogen base with ammonium sulfate without amino acids.

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tem have been made. Bacterial expression systems have often failed to produce high yields of properly folded cystine-rich proteins such as PBPs. Nevertheless, with three PBPs, approximately 5% of the proteins expressed in bacteria could be obtained in soluble forms, purified and characterized as functionally active (10). Moreover, PBPs expressed in inclusion bodies have been successfully solubilized, refolded into their pheromone-binding native forms (7, 11, 12). PBP from the male silkmoth *Antheraea pernyi* has been also expressed by baculovirus-infected insect cells but leading to a low level of secreted protein (13).

The *Pichia pastoris* yeast expression system provides an excellent alternative and has been successfully used to produce large quantities of disulfide-bonded proteins (14, 15). Secretion is the best mode of protein production since the organism secretes only very low levels of its own proteins which facilitates protein purification. The secreted protein requires the presence of a signal sequence to target it into the secretory pathway. The *P. pastoris* acid phosphatase (PHOI) secretion signal is not preferred since, after secretion, it leaves an amino acid (Arg) at the N-terminus of the recombinant protein. Two recent studies concerning the recombinant β-cryptogein and the synthetic monocyte chemoattractant protein 1 showed that the presence of an extra Arg at the N-terminus or the lack of the first two amino acids of the protein could significantly change its biological activity (16, 17). The 89 amino acid secretion signal sequence from *Saccharomyces cerevisiae* α-factor prepropeptide has been used with the most success (18) such as, for instance, aprotinin (19), mouse epidermal growth factor (20), thrombomodulin fragment, blood factor XII (14), a fragment of amyloid β-protein (21), antibody single-chain Fv fragment (22), N-lobe of human serum transferrin (23), human interleukin-17 (24), hirudin (25), and anticoagulant–antimetastatic protein ghilanten (26). However, even with this signal peptide, aberrant processing has been observed (27, 28).

As an alternative, the native signal from different organisms has also been used successfully for heterologous protein expression in *P. pastoris* (29–34). In this paper, we examined the impact of the *S. cerevisiae* α-factor preprosequence with and without the Glu-Ala repeats or the insect signal peptide on the secretion of properly processed and folded ASP2 into a minimum buffered salt medium. The purification and the biochemical characterization of the recombinant protein ASP2 are also described.

MATERIALS AND METHODS

Materials

The pHIL-D2 and pPIC9 shuttle vectors and *P. pastoris* strain GS115 (his4$^-$) were from Invitrogen (The Netherlands). Media components were purchased from Difco (Serlabo, France). *Escherichia coli* strain DH5α was used for DNA subcloning. Restriction endonucleases were purchased from Eurogentec (Belgium). All other reagents were obtained from Sigma (France). ASP2 cDNA clone O12.7 (8) was used as the template for polymerase chain reaction (PCR).

Construction of Expression Vectors

The following different expression constructs were made as outlined in Fig. 1.

(a) The cDNA encoding the mature ASP2 without its native signal peptide was amplified by PCR using the following primers: 5′ primer, 5′-TGTCTGCTACTCGAGAAAAGAGAGGCTGAAGCTATAGATCAAGACACCGTAGTC-3′; 3′ primer, 5′-ATACGTGAATTCTTACGAGAACAGTTTCTCGAT-3′ and the ASP2 cDNA clone O12.7 as the template. The PCR-amplified fragment was cloned into the *Xho*I and *Eco*RI sites of pPIC9, generating the construct with the full preprosequence pEAEAαASP2. In this construct, two Glu-Ala repeats have been introduced between the α-factor mating prepropeptide and the mature ASP2 sequence.

(b) The 5′ primer used in the first construct was replaced with the 5′ primer, 5′-GGATCGCTCGAGAAAAGAATAGATCAAGACACC-3′ to delete the two Glu-Ala repeats between the α-factor secretion peptide and the mature ASP2 sequence. The PCR-amplified fragment was cloned into the *Xho*I and *Eco*RI sites of pPIC9, generating the construct pαASP2.

(c) In the last construction pNatASP2, the cDNA encoding the native precursor ASP2 was amplified by PCR using the following primers:the 3′ primer used in the first two constructs and the 5′ primer, 5′-CAACTTGAATTCACCATGAACACCCTCGTCACC-3′ allowing the creation of a Kozak consensus sequence and the restriction sites. The PCR-amplified fragment was cloned into the *Eco*RI site of pHIL-D2 (previously cleaved by *Eco*RI and treated with calf intestinal phosphatase to reduce self-ligation). The correct orientation of the DNA insert was determined by PCR using the 5′ *AOX1* primer from Invitrogen and the 3′ primer described in (a).

DNA Sequencing

The dideoxy chain termination method of DNA sequencing was carried-out on double-stranded DNA plasmids with BigDye terminators (Applied Biosystems, France) on a PCR apparatus (DNA thermal cycler 480, Perkin–Elmer, France). The PCR products were analyzed on an automatic sequencer (ABI PRISM 310, Applied Biosystems).

Transformation of P. pastoris

Yeast transformation was performed using a spheroplasting method as described in the manual (Version 3.0) of the *Pichia* expression kit (Invitrogen). For transformation, the plasmids pEAEAαASP2 and pαASP2 were digested with *Bgl*II and pNatASP2 with *Not*I. After 4–6 days of incubation at 29°C on RDB medium (1 M sorbitol, 2% w/v D-glucose, 1.34% w/v YNB, 4 μg/ml D-biotin, 0.005% v/v amino acids without histidine), isolated colonies were streaked onto MM (0.5% v/v methanol, 1.34 w/v YNB, 4 μg/ml D-biotin) and MD (2% w/v D-glucose, 1.34% w/v YNB, 4 μg/ml D-biotin) agar plates. After 2 days, Mut^S transformants (slow growth on methanol as a sole carbon source) were retained for further study.

Selection of Secreting Clones

His^+ Mut^S transformants were inoculated in 10 ml of BMGY medium (1% w/v yeast extract, 2% w/v peptone, 1.34% w/v YNB, 4 μg/ml D-biotin, 100 mM potassium phosphate, pH 6.0, 1% v/v glycerol) in sterile 50 ml Erlenmeyer flasks which were then incubated at 29°C, 300 rpm for 2 days. Induction of protein expression was achieved by harvesting the cells by centrifugation at 3000*g* for 5 min at room temperature and resuspending them in 2 ml of BMM medium (1.34% w/v YNB, 4 μg/ml D-biotin, 100 mM potassium phosphate, pH 6.0, 0.5% v/v methanol) before continuing the incubation as earlier. After 2 days, samples were centrifuged at 12,000*g* for 10 min at room temperature to pellet the cells and 100 μl of the supernatant was removed for analysis by automated HPLC on a HP1090M device (Hewlett–Packard, France). Recombinant ASP2 was analyzed by reversed-phase HPLC on a Aquapore (C8) RP 300 column (0.21 i.d. $\times$ 3 cm, Brownlee, Perkin–Elmer, France) equilibrated with solvent A (0.1% TFA in H_2O) and eluted with a linear gradient up to 60% solvent B (95% acetonitrile, 5% H_2O, 0.085% TFA) for 30 min. The temperature of the column was maintained at 40°C. The flow rate was 0.2 ml/min and the absorbance was recorded at 215 nm.

Gel Electrophoresis

SDS–PAGE (16% acrylamide) was performed using a Mini-Protean II system (Bio-Rad, France) according to the method of Schägger and Von Jagow (35) with modification (36). Sample preparation consisted of the centrifugal evaporation of 10 μl of each culture supernatant before resuspension of the samples in 20 μl of sample buffer (12% v/v glycerol, 4% w/v SDS, 3% w/v dithiothreitol, 50 mM Tris–HCl, pH 6.8). The molecular weight calibration kit PMW (Pharmacia, France) was used and the proteins were visualized by Serva blue G staining.

Ion Spray-Mass Spectrometry (IS-MS)

Microion spray-mass spectrometry was performed on a Sciex API100 (Perkin–Elmer, France) using a positive mode from 1200 to 3000 a.m.u. with 0.1 a.m.u. steps and a 1 ms dwell-time. The ion spray needle voltage was 5000 V and the orifice plate voltage +40 V. Infusion was performed at a flow rate of 0.2 μl/min. The quadrupole was calibrated with a Perkin–Elmer polypropylene glycol standard solution. Mass spectrometry data were analyzed with the Perkin–Elmer–Sciex Bio-Multi-View 1.2 software. The average molar masses were calculated using the Perkin–Elmer–Sciex Peptide Map 2.2 software.

Edman Degradation

N-terminal amino acid sequence analysis of proteins was performed by automated Edman degradation using a Perkin–Elmer Procise 494-610A protein sequencer with reagents and methods of the manufacturer.

Thiol Titration

To quantify the extent of free thiols of the recombinant ASP2, the color reaction using 5,5′-dithiobis (2-nitrobenzoic acid) developed by Ellman (37) was performed. Tenfold excess of Ellman's reagent over protein was used and the number of reactive cysteine residues was quantified by following the absorbance at 412 nm.

Shake-Flask Production

Having identified the best protein-producing pNatAsp2 transformants and determined the optimal growth pH after induction, intermediate-scale culture was carried out using an incubator Aerotron (Infors, France). Several colonies of a single isolate were used to inoculate 50 ml of BMGY in a 500-ml baffled flask which was then incubated at 29°C, 300 rpm, continued for 16 h; 20 ml of this preculture was then used to inoculate 2 liters of BMGY divided between four 3-liter baffled flasks. Incubation at 29°C, 300 rpm, was continued for 2 days and at the end of this first phase of fermentation, the cell density reached about 100 g (wet weight) of cells per liter of culture. The cells were pelleted at 3000*g* for 20 min at 4°C and the supernatant was discarded. The cells were resuspended in a total of 1 liter of BMM medium, pH 7.0, in four sterile 3-liter baffled flasks covered only with one layer of Scrynel NYHC nylon (100-μm mesh, Polylabo, France) and incubation at 29°C, 300 rpm, was continued for 7 days. During the induction period, methanol was fed every 24 h to maintain a concentration of 0.5% v/v. About 7 days onward, the level of recombinant protein secreted into the culture medium reached a plateau.

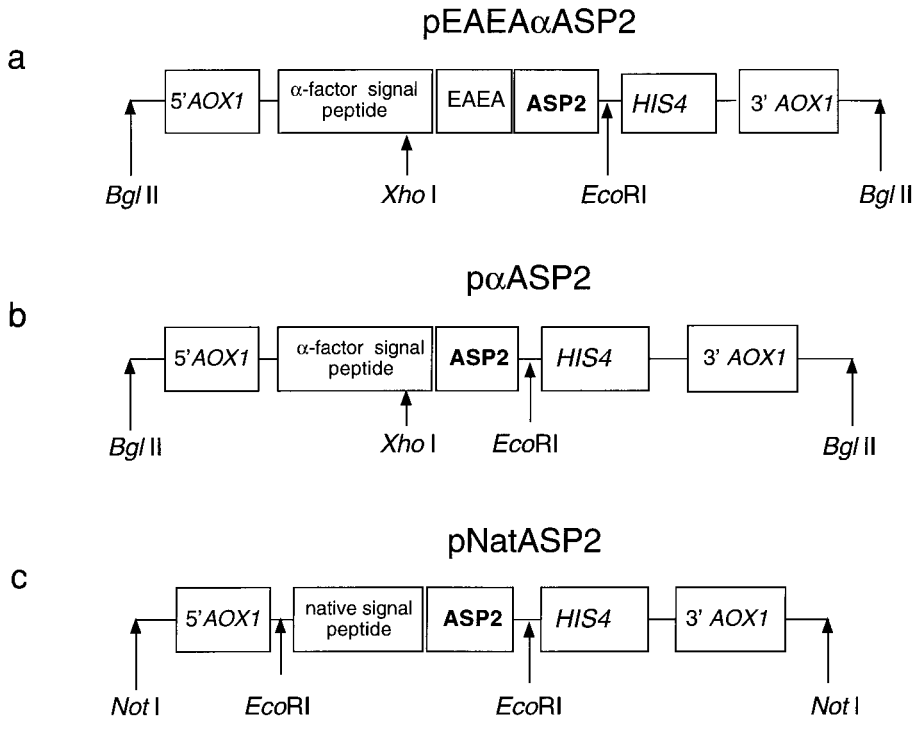

FIG. 1. Construction of the expression vectors for ASP2. The linearized vectors are composed of the *AOX1* promoter (5′ *AOX1*), the mature ASP2 cDNA, the histidinol dehydrogenase gene (*HIS4*) for selection of transformants and the 3′ *AOX1* sequence from the *AOX1* gene. (a) The coding mature portion of ASP2 cDNA was cloned between *Xho*I and *Eco*RI sites of the expression vector pPIC9 in frame with the α-mating prepro-factor signal peptide with the spacer peptide Glu-Ala-Glu-Ala. (b) The coding mature portion of ASP2 cDNA was cloned between *Xho*I and *Eco*RI sites of the expression vector pPIC9 in frame with the α mating prepro-factor signal peptide without the Glu-Ala repeats. (c) The coding region of ASP2 cDNA with its native peptide sequence was cloned into the *Eco*RI site of the expression vector pHIL-D2.

The cells were removed by centrifugation at 10,000*g* for 30 min at room temperature and the supernatant was recovered.

Purification of Recombinant ASP2

ASP2 has been purified by anion-exchange chromatography. After removing insoluble components from supernatant containing ASP2 by filtration, the solution was equilibrated with 10 mM Tris–HCl at pH 8.0 by dialysis for 4 days at 4°C, using a dialysis tube with 8000 Da cutoff (Servapor, Polylabo, France). The chromatographic conditions have been first determined using a Vydac QAE column (300 VHP, 0.75 i.d. × 5 cm, Interchim, France). After loading the dialyzed supernatant, the column was washed with 10 mM Tris–HCl at pH 8.0 and the elution was achieved using a linear gradient with the same buffer to 0.5 M NaCl in the first 60 min and to 1 M NaCl in the next 20 min. The flow rate was 0.5 ml/min and the absorbance at 280 nm was recorded. The purification was performed using two QAE Zeta-Prep (disk 60) disks (Cuno, FLOT, France) using a peristaltic pump with a flow rate of 2.5 ml/min at room temperature. Disks were equilibrated in 10 mM Tris–HCl at pH 8.0. After loading the supernatant, the disks were washed with the same buffer containing 0.2 M NaCl. The elution was performed with the Tris buffer containing 0.45 M NaCl and monitored at 280 nm. The fractions containing purified ASP2 were pooled, dialyzed extensively against Milli-Q H_2O, and lyophilized.

RESULTS AND DISCUSSION

Construction of Expression Vectors and Transformation into P. pastoris

To compare the production level and the posttranslational processing in *P. pastoris,* we constructed three different *Pichia* expression plasmids. Since it was not certain whether the insect signal peptide could direct secretion of ASP2 from the yeast cell, we also secreted ASP2 with the yeast prepropeptide signal from the *S. cerevisiae* α-mating factor. pEAEAαASP2 and pαASP2 vectors were constructed by inserting the mature coding sequence of ASP2 downstream from the methanol-inducible alcohol oxidase (*AOXI*) promoter between the *Xho*I and *Eco*RI restriction sites (Fig. 1). The protein ASP2 was also expressed with its natural honeybee signal peptide using the pHILD2 vector. The full coding cDNA sequence, including the natural signal pep-

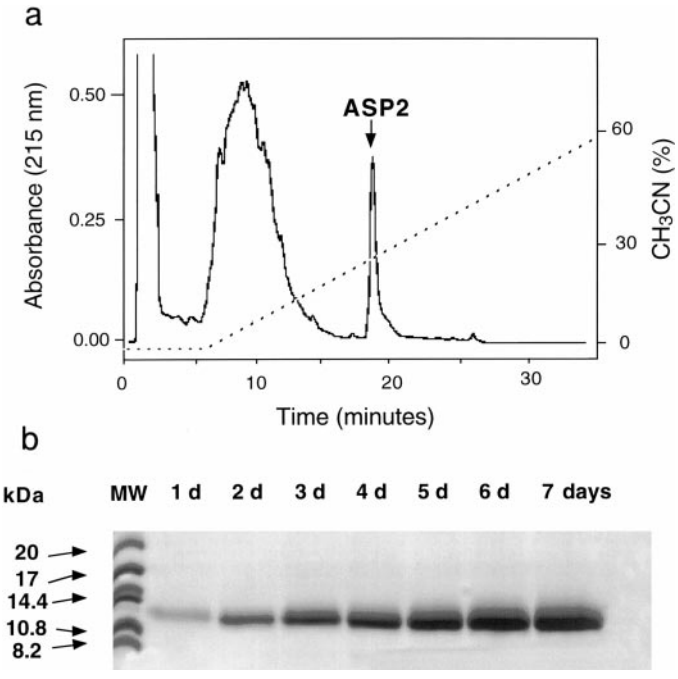

FIG. 2. (a) HPLC analysis of the culture supernatant of the best protein-producing pNatAsp2 *P. pastoris* transformant. The expression supernatant was chromatographed on an Aquapore (C8) RP 300 column (0.21 i.d. × 3 cm, Brownlee, Perkin–Elmer, France) equilibrated with solvent A (0.1% TFA in H_2O) and eluted with a linear gradient (dashed line) up to 60% solvent B (95% acetonitrile, 5% H_2O, 0.085% TFA) in 30 min. (b) SDS–PAGE analysis of recombinant ASP2 secreted by *P. pastoris* using its native peptide signal. Lane 1 shows standard (kit PMW, Pharmacia, France) and lanes 2 to 8 are 10-μl aliquots of a 1- to 7-day culture supernatant.

tide and a Kozak sequence introduced by PCR, was inserted at the unique *Eco*RI site flanked by the 5′ and 3′ regulatory sequence of *AOXI* promoter giving rise to pNatASP2 vector. For transformation, the plasmids pEAEAαASP2 and pαASP2 were digested with *Bgl*II and pNatASP2 with *Not*I to give an integrative fragment containing the ASP2 cDNA and the *HIS4* selectable marker. Approximately 300 His^+ transformants of the GS115 strain were obtained by spheroplasting. Fifty transformants corresponding to Mut^s phenotypes were isolated. They were grown in the BMGY medium and induced at 29°C for 2 days in the BMM medium buffered to pH 6.0. The higher producing clones were screened by determining the amount of ASP2 secreted in the extracellular medium by automated HPLC (Fig. 2a). The recombinant protein was found to be the major component and eluted at 29% acetonitrile as the native protein (8). Samples of the expression medium supernatants, taken at various time intervals, were also analyzed by SDS–16% polyacrylamide gel to determine the optimal induction time. Only the recombinant protein, migrating at about 14 kDa, was detectable by Serva blue G staining. The electrophoretic profile (Fig. 2b) reveals that the protein regularly accumulates up to approximately 0.1 mg/ml over an expression period of 7 days, while other proteins were present in trace amounts only.

Influence of the Signal Sequence on the Secretion of ASP2 by P. pastoris

The preprosecretion signal of *S. cerevisiae* α-mating factor has already been used successfully to secrete heterologous proteins in *P. pastoris*. In *S. cerevisiae*, it has been noted that the preprosequence of α-mating factor is cleaved by the *Kex2* gene product in the Golgi apparatus and that the Glu-Ala repeats were further cleaved by the diaminopeptidase encoded by *Ste13*. It has been observed that the Glu-Ala repeats were not necessary for cleavage by Kex2 but increase the cleavage efficiency. The Glu-Ala-Glu-Ala spacer sequence is subsequently cleaved by the diaminopeptidase Ste13 supposed to leave the protein of interest free of additional N-terminal residues. Therefore, we compared the secretion of the recombinant protein using either the preprosequence of the α-mating pheromone of *S. cerevisiae* with or without the Glu-Ala-Glu-Ala spacer peptide.

Depending on the signal sequence used, N-terminal sequencing of HPLC-purified ASP2 revealed multiple termini as indicated in Fig. 3. Based on the amount of residues released in each cycle of Edman degradation, the percentage of the different N-terminal forms of ASP2 has been determined. While the prepropeptide of the α-factor with the spacer leads to a mixture of heterogenous proteins with multiple N-termini, we found that the preprosequence without the Glu-Ala repeats was efficient for proper secretion and cleavage of recombinant ASP2 in *P. pastoris*.

To maximize production of ASP2, we also expressed the protein using its own natural leader peptide. The cDNA encoding the native precursor ASP2 was cloned into the *Eco*RI site of pHIL-D2 and used to transform the yeast cells. Correct processing of the signal sequence was verified by N-terminal analysis of HPLC purified ASP2. The first 12 residues were in agreement with the native ASP2 amino acid sequence (Fig. 3). The natural insect signal peptide was then revealed to be efficient for proper secretion of heterologous ASP2 in *P. pastoris*. The best producing clone for each of the three constructions secreted the recombinant ASP2 at a similar level of 150 mg/liter.

Integrity of Recombinant ASP2

Whatever the construction, a proteolytic C-terminal degradation of the protein ASP2 was also observed, probably caused by extracellular proteases during the fermentation at pH 6.0. IS-MS showed an amputation of 7 C-terminal amino acids of the protein (data not

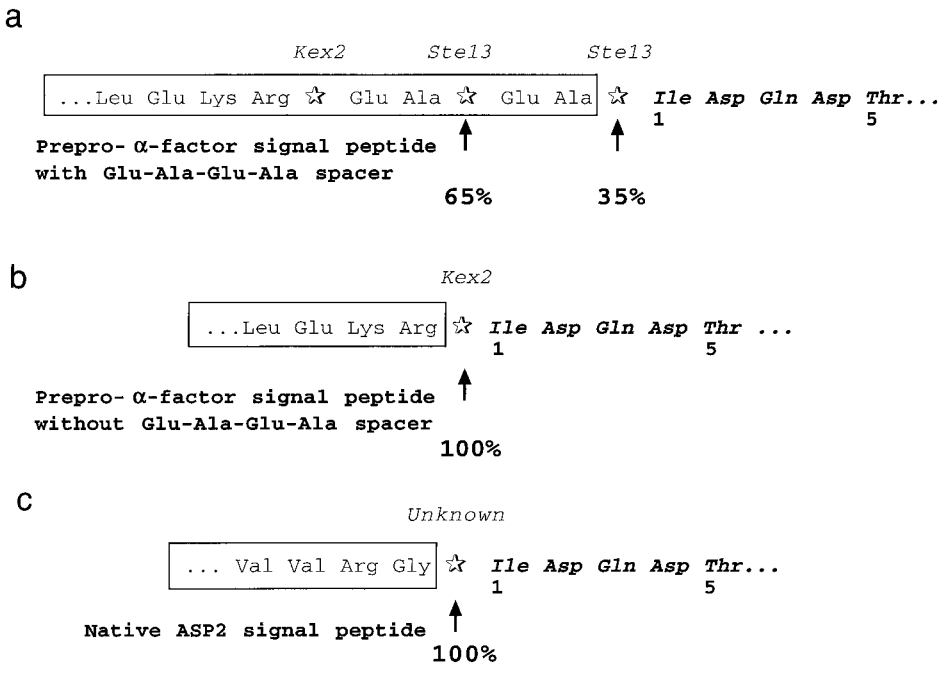

FIG. 3. Determination of N-terminal amino acid sequences of ASP2 secreted using the different signal peptides. The processed N-termini are indicated in bold italics; the removed signal sequences are boxed. The cleavage sites processed by the proteases encoded by *Kex2* and *Ste13* genes are indicated by a star. (a) α-mating prepro-factor signal peptide with the spacer peptide Glu-Ala-Glu-Ala, (b) α-mating prepro-factor signal peptide without the Glu-Ala repeats, (c) natural honeybee peptide sequence. The percentages of each secreted ASP2 forms are shown under the arrows indicating the corresponding N-termini.

shown). Proteolysis was nevertheless observed to be affected by the pH of the culture. Since *P. pastoris* cells can grow in a wide variety of pHs ranging from 3 to 9, we therefore maintained the culture medium at pH 7.0–7.5 during cell induction to inactivate the endogenous proteases.

IS-MS on the recombinant protein secreted using its native leader sequence (Fig. 4) showed a predominant peak, together with derivatives corresponding to Na and K adducts. The ASP2 mass was found to be 13,695.1 ± 1.7 Da which is in perfect agreement with the measured molecular mass (13,695.2 ± 1.6 Da) of the native honeybee protein (8) which is known to not undergo any posttranslational modification other than the cleavage of the 19-residue signal peptide and the formation of three disulfide bridges. The sulfhydryl titration using the method of Ellman confirmed that the three disulfide bridges were indeed formed since a content of approximately 0.1 thiol/protein was measured. The yeast secretion machinery is therefore able to properly process secretory proteins with an insect peptide signal without proteolytic degradation in our culture conditions.

Shake-Flask Production of ASP2 in P. pastoris

The clone with the highest level of ASP2 expression using the natural peptide was chosen for intermediate-scale production in fermentation culture in BMM medium maintaining the pH at 7.0–7.5. The expression volume has been scaled up to 250 ml per flask and the cell concentration in the expression phase was stable at 100 g wet weight cells per liter of culture. The purified ASP2 production reached a level of 150 mg/liter over an

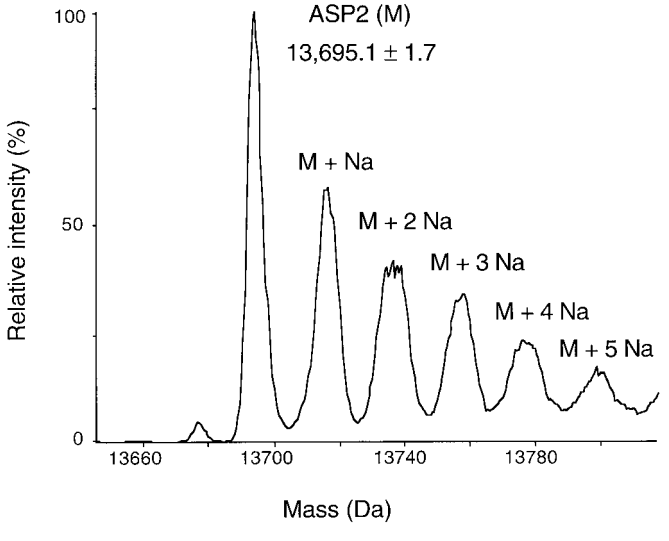

FIG. 4. Reconstructed ion spray mass spectrum of ASP2 with Na and K adducts.

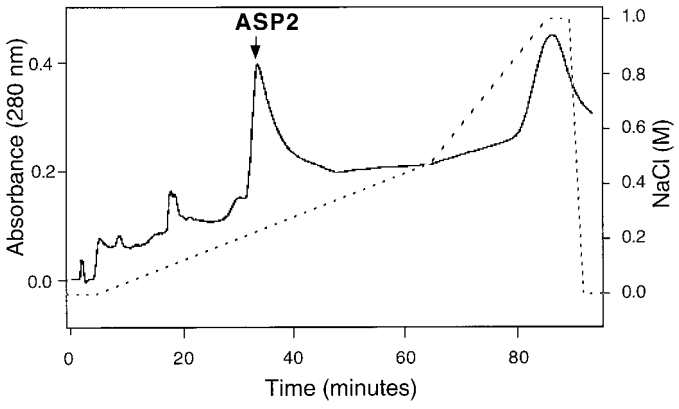

FIG. 5. Chromatogram of ASP2 purification from the cell culture supernatant by ion-exchange chromatography. The expression supernatant was chromatographed on a Vydac QAE column (300 VHP, 0.75 i.d. × 5 cm, Interchim, France). ASP2 was eluted with a NaCl gradient (dashed line) from 0 to 0.5 M NaCl in the first 60 min and to 1 M NaCl in the next 20 min in 10 mM Tris–HCl, pH 8.0.

expression period of 7 days. Moreover, efficient expression and secretion could be repeated by reincubation of the isolated cells in minimal medium for 7 more days.

Purification of the Recombinant Protein ASP2

The high-level secretion of ASP2 from *P. pastoris* facilitated the development of a simple purification method. To determine the chromatographic conditions, after centrifugation at 10,000*g* for 30 min, the supernatant was clarified by filtration, submitted to dialysis, and then applied to a Vydac QAE column (Fig. 5). Elution was achieved using a linear gradient with 10 mM Tris–HCl at pH 8.0 from 0 to 0.5 M NaCl in the first 60 min and to 1 M NaCl in the next 20 min. The protein was eluted as a single peak at relatively high ionic strength (0.25 M NaCl) in agreement with the calculated p*I* and migrated as a single 14-kDa species on SDS–PAGE as expected (data not shown).

The purification was performed using two QAE disks equilibrated in 10 mM Tris–HCl at pH 8.0. After loading the supernatant, the disks were washed with the same buffer containing 0.2 M NaCl. The elution was performed with the Tris buffer with 0.45 M NaCl. N-terminal sequencing, IS-MS, and reversed-phase chromatography confirmed that the recombinant protein ASP2 was greater than 96% pure and in perfect agreement with expected features. This purification procedure produced over 150 mg of purified ASP2 from the 1-liter culture supernatant with 85% overall yield.

CONCLUSION

The *P. pastoris* expression system was chosen to express ASP2 because it allows to produce large quantities of soluble disulfide-bonded proteins. This system efficiently secreted ASP2, properly removed the natural signal sequence, and allowed the formation of disulfide bonds, without unexpected processing provided the culture medium was maintained at a pH ranging from 7.0 to 7.5. The produced protein is very pure and easy to purify since *P. pastoris* secretes only very low levels of its own proteins in the medium. The level of expression that we obtained, approximately 150 mg/liter, is about 100-fold the expression of an insect PBP using baculovirus-infected insect cells (13). To our knowledge, this is the first insect OBP being highly expressed in soluble form with correct disulfide bond formation.

The recombinant protein ASP2 produced in high amounts should allow the screening of specific odorants and the study of their binding properties. In addition, this expression system, easy to scale-up, provides sufficient material for the crystallization trials which are currently under way. Finally, this expression system opens the possibility for site-directed mutagenesis of specific residues to investigate and clearly define the relationships between the structure and the function of this novel protein.

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